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THE PHYSIOLOGICAL ECOLOGY AND EVOLUTION OF FREEZING TOLERANCE IN DIPLOID LARREA SP.

by

Juliana Sibylla Medeiros

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

December, 2009

This dissertation is dedicated to the memory of my grandparents

J.N. “Norm” Brand

who showed me that kindness, hard work and pride in a job well done

can make even an ordinary life extraordinary

and

Barbara Jean “B.J.” Brand

whose tenacious, adventurous and philosophical spirit lives on in my work

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The completion of field work abroad would not have been possible without an amazing number of Mexican and Argentine researchers who generously provided their

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By

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ABSTRACT

A basic understanding of vegetation responses to temperature and precipitation variability is paramount to the understanding of ecological adaptation, and comparisons across warm deserts are needed to increase our understanding of the controls on establishment in warm desert ecosystems. At the high latitude edges of warm deserts drought and freezing lower plant performance due to xylem dysfunction, as freeze-thaw embolism reduces stomatal conductance, or damage to leaf photosynthetic apparatus, as tissue desiccation reduces photosynthetic efficiency. Variation in these physiological responses can be observed across life-history stages, genetic background, climate gradients, and species, and this variation may be acted on by selection to produce adaptation to local climate conditions.

The primary goal of our research was to determine the interactive effects of freezing and drought on the performance and survival of high latitude plants and to determine if physiological and morphological adaptations to freezing exist in populations which experience freezing. Data were collected from greenhouse and field grown plants from high and low latitude populations in North and South America. We observed differences in physiological and morphological characters across latitude for both *L.*

tridentata and *L. divaricata,* in the absence of differences in ploidy, indicating that local adaptation to freezing has occurred in the warm desert shrub genus *Larrea*. We observed correlations between freezing tolerance and minimum temperatures accompanied by higher freezing tolerance in high latitude populations, suggesting that the evolution of increased freezing tolerance could have facilitated recent high latitude expansions of *Larrea* populations. Heritable variation is present in both high latitude *L. tridentata* and low latitude *L. divaricata* populations, allowing the potential for these populations to respond to climate change. The high latitude *L. divaricata* population exhibited low levels of variation, which may have resulted from prior selection for increased freezing tolerance and reduces the rate of evolution of freezing tolerance. Finally, fluctuating selection may maintain variability in seedling traits in the high latitude *L. tridentata* population. In the presence of mild drought, survival and performance following freezing events may by enhanced, and establishment of seedlings could be expected to occur in either warm, wet or cold, dry winters.

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CHAPTER ONE

INTRODUCTION

Although water availability probably limits the distribution of many arid-land species, freezing also occurs at the high latitude edges of warm deserts and may exert additional pressures on the expansion of desert evergreen populations (Martinez-Vilalta & Pockman 2002; Ewers *et al.* 2003). At high latitude edges of deserts, plants typically experience the coldest temperatures within their distributions, drought and freezing often occur together, and interactions between freezing and drought stress may occur (Pratt *et al.* 2005; Mayr *et al.* 2006a). The potential for low temperature stress to limit plant performance and survival, however, depends on the effects of drought on freezing tolerance and whether or not populations at high latitudes have or can develop adaptations to freezing. Adaptations to freezing have been observed in trees (Tyree & Cochard 1996; Nardini *et al.* 2000; Feild & Brodribb 2001) and shrubs (Noshiro & Baas 2000; Cordero & Nilsen 2002; Ewers *et al.* 2003). Plants can show plastic responses to low temperature stress, changing their physiology and/or morphology, which can result in population differentiation without genetic differentiation (Fisher *et al.* 2007).

WHAT ARE THE PHYSIOLOGICAL EFFECTS OF FREEZING?

Living cells such as those in the leaves contain solutes which lower the freezing point of these tissues. This phenomenon is known as super-cooling and can protect cells from freezing at least 7^{o}C below zero. When living cells are protected, the water-containing tissues surrounding leaves are the first to freeze (Sakai & Larcher 1987). The formation of ice in the apoplast reduces plant water potential in these tissues, and this causes the dehydration of living cells. When freezing finally occurs in living cells the growth of ice can cause mechanical damage, disrupting membranes (Pearce 2001; Ball *et al.* 2004).

In addition to damaging living cells, freezing can also negatively impact water transport from soil to leaves (Cochard & Tyree 1990; Sperry & Sullivan 1992; Tyree & Cochard 1996) as gases, which are less soluble at lower temperatures, form bubbles in xylem conduits as xylem sap cools. If transpiration resumes before these bubbles re-dissolve the bubbles may expand, resulting in a xylem embolism and reducing water transport to the leaves. The size of the xylem conduit determines how large these bubbles may become, and so larger conduits are more vulnerable to freezing embolism (Sperry & Sullivan 1992; Davis *et al.* 1999; Pitterman & Sperry 2003). Emboli can be refilled, but this is likely dependant on living cells associated with xylem (Ball *et al.* 2006; Cavender-Bares *et al.* 2005).

WHAT ARE THE EFFECTS OF DROUGHT ON FREEZING TOLERANCE?

The water status of a plant can have a large impact on vulnerability to freezing, but the effects on leaves and xylem are often different (Feild & Brodribb 2001; Cavender-Bares *et al.* 2005; Coopman *et al.* 2008). In the leaves and other living cells, the physiological damage caused by drought and freezing are similar; both cause dehydration and can result in mechanical damage to cell membranes (Xin & Browse 2000). During drought cells accumulate solutes to reduce water loss and protect cellular organelles, additionally drought induces the up-regulation of stress responses, which can provides some protection from the damaging effects of freezing. Physiologically, drought and cold acclimation are similar, and may both cause the up-regulation of the same stress related genes (Horvath *et al.* 2007; Bouchabke-Coussa *et al.* 2008; Pasquali *et al.* 2008). Like drought, cold acclimation also induces changes in cell physiology, membrane properties and gene regulation (Xin & Browse 2000).

In xylem the opposite is true, and the presence of drought negatively affects freezing tolerance. As bubbles form in the xylem during freezing the xylem water potential determines the likely-hood that bubbles will spontaneously expand to form an embolism, so as water potential drops, smaller and smaller bubbles can result in embolism (Davis *et al.* 1999; Ewers *et al.* 2003; Willson & Jackson 2006). If xylem is more vulnerable at low water potential and leaves are less vulnerable this could result in a mismatch of water supply and demand (Sperry *et al.* 2002). Smaller conduits are safer, but larger conduits are more efficient and plants often face a trade-off between safety and efficiency when producing xylem vessels (Sperry *et al.* 2008). Maximization of growth during times of high water availability while tolerating some loss of water transport to freezing embolism may effectively maximize growth over the long term (Carlquist 2001).

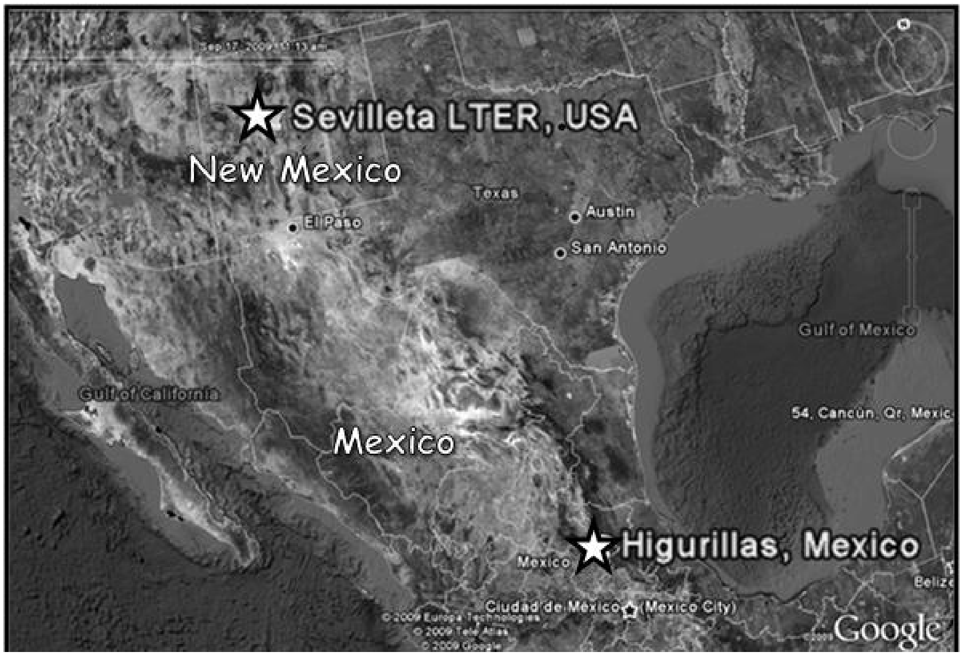
IS THERE EVIDENCE FOR LOCAL ADAPTATION TO FREEZING IN THE GENUS *LARREA*?

Evidence that selection has acted in the past includes the observation that variation in physiology and morphology exists across populations, and latitudinal variation in freezing tolerance has been previously reported (Ewers *et al.* 2003; Feild & Brodribb 2001). In addition, because both plasticity and adaptation can result in population differentiation, separation of environmental from genetic components of variation can determine the relative contribution of genetic vs. plastic variation. A strong genetic component to variation has been detected in the wood characters of several species (St. Clair 2006; Zhen & Ungerer 2007; Liu & Noshiro 2003). Plastic responses can also be inherited, such as cold acclimation, a trait which has also been shown to vary across populations (Loik & Nobel, 1993; Nardini *et al.* 2000).   However, other studies have detected a strong effect of environment on wood characters (February & Manders 1999; Leal *et al.* 2003; Stevenson & Mauseth 2004; Aguilar-Rodriguez *et al.* 2006; Thomas *et al.* 2007) and evidence that further freezing tolerance could be selected must include data on the structure of variation in physiology and morphology within populations. Selection can only result in evolution of increased freezing tolerance if there is heritable variation in the character within populations. Freezing tolerance is well known to vary within populations of crop species (Aguilera *et al.* 1999; Rapp & Junttila 2001; Jank *et al.* 2002), and this is the basis for breeding more freezing tolerant varieties. Some data on conifers suggests that freezing tolerance is heritable within natural populations (St Clair 2006), but nothing is known about the within-population variation in this trait among warm desert species. Common garden experiments which compare variation in physiology, survivorship, size or reproduction within and across populations can reveal the relative contribution of plastic vs. genetic effects and provide evidence for or against local adaptation (McGraw & Wulff 1983; Ackerly *et al.* 2000; Arntz *et al.* 2000).

THE GENUS *LARREA* IN NORTH AND SOUTH AMERICA

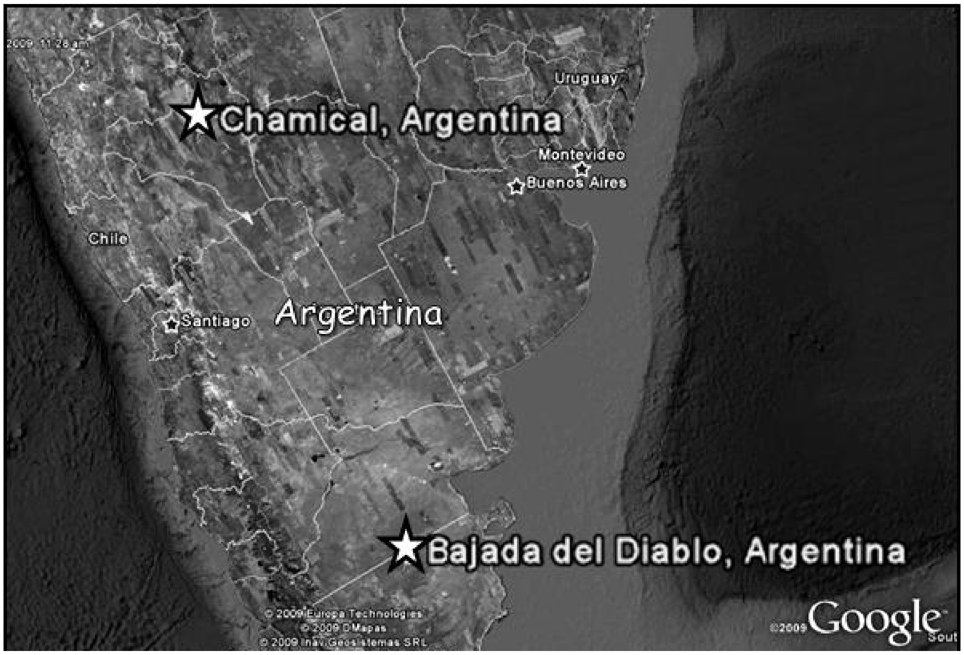
The genus *Larrea* provides an excellent system in which to study the physiological ecology and evolution of freezing tolerance in warm desert species. The evergreen shrub genus spans a wide range of latitudes throughout the warm deserts of both North and South America where freezing is common at high latitudes but rare and less intense at low latitudes within the distribution (Mabry *et al.* 1977). Evergreens may be particularly vulnerable to stochastic freezing events because they maintain physiological function during winter.

Figure 1-1. High and low latitude field sites in North America. *The high latitude site is located in the state of New Mexico, USA at the Sevilleta LTER. The low latitude site is located in the state of Queretaro, Mexico near the town of Higurillas. Image from Google Earth.*



One species, *L. tridentata*, is distributed widely across the three warm deserts of North America, the Mojave, the Sonoran and the Chihuahuan (Henrickson & Johnston 1997; *Figure 1-1*). Differences in ploidy exist across the three deserts, with Mojave desert *L. tridentata* being hexaploid, Sonoran populations are tetraploid and Chihuahuan desert populations are diploid (Barbour 1969; Duran *et al.* 2005). In South America there are four species of *Larrea* which occur in the warm deserts of Argentina (*Figure 1-2*). One of these, *L. cuneifolia*, occurs only in the hottest, driest regions of the Monte Desert in Argentina and is therefore not likely to have any special adaptations to freezing. A second, *L. ameghinoi* occurs in cold, dry areas of Patagonia, but its prostrate growth form likely allows it to escape freezing. Two other species occur in the Monte desert in Argentina, *L. divaricata* and *L. nitida*. *L. divaricata* is most similar in physiology and ecology to its North American cousin *L. tridentata* (Barbour 1969), and the two have only been recently distinguished as separate species. *L. divaricata* is widespread in the Monte desert and its range includes coastal deserts and mountain foothills across a wide range of latitudes. *L. nitida* is restricted to colder upland habitats, and so might be expected to have some adaptations to freezing (Ezcurra *et al.* 1991).

Figure 1-2. High and low latitude field sites in South America. *The high latitude site is located in the state of Chubut, Argentina near the town of Bajada del Diablo. The low latitude site is located in the state of La Rioja, Argentina near the town of Chamical. Image from Google Earth.*



Previous work investigating the freezing tolerance of *L. tridentata* has shown differences between tetraploid Sonoran and diploid Chihuahuan desert populations, with the xylem of high latitude Chihuahuan desert populations being more freezing tolerant than lower latitude Sonoran desert populations (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002). Polyploidy is known to cause an increase in cell size, however, and because xylem freezing vulnerability is closely related to cell size, the effects of ploidy and freezing were confounded in these previous studies. In addition, nothing is known about the freezing tolerance of the South American members of this genus.

It is well known that plants from regions of similar climate across the world display striking similarities in adaptation, despite genetic isolation, the warm deserts of North and South America being no exception (Barbour 1969; Orians & Solbrig 1977). However, until now, no study had specifically addressed convergence in the eco-physiology of freezing and drought across the large latitudinal gradient of populations of North and South American warm deserts. Since studies of the physical and biotic environments of these two species were conducted in the 1970's (Orians & Solbrig 1977; Mabry *et al.* 1977) little comparative research had been done across these biomes, especially at the northern edge of Argentina.

Vegetation responses to temperature and precipitation variability offer insight into ecological adaptation (Comstock & Ehleringer 1992), and comparisons across warm deserts are needed to increase our understanding of how species might respond to climate change in warm deserts across the globe. The interactive effects of temperature and water availability on individual performance and survival are likely crucial in shaping the distribution of this dominant warm desert species, but many of the details of these relationships are at present, unknown. Indeed, just as past climate changes have resulted in the patterns we observe today, the warmer, wetter winters predicted by global climate change models are likely to change the relative abundance of desert scrub across North and South America (Bachelet *et al.* 2001; Epstein *et al.* 2002; Peters 2002).

The complicated interactions and large number of unknowns inherent in questions concerning plants in their natural environments necessitates a multi-level approach which utilizes independent measurement techniques. Laboratory freezing experiments provide quantitative data on the relative vulnerability of different individuals and different plant tissues, while measurements of the variation in physiological traits within and among populations under field and greenhouse conditions can provide valuable information as to the specific vulnerability under naturally occurring conditions. The validation of conclusions through the use of varied methods can help unravel the many factors that determine the physiological state and responses of plants under stress. The importance of these findings are not dependent upon a particular climate change scenario because we expect that the limits and interactions that we have quantified will help predict the response of this widespread genus to a range of changes in drought and freezing regimes across their distribution. We compared physiological adaptations and responses to the interaction of drought and freezing in a high latitude population of *L. tridentata* in North America. We also conducted a field survey of the wood anatomy of adults at the high and low latitude populations to determine if populations of *Larrea* species exhibit adaptations to local climate. Finally, we investigated the variation in physiological responses to freezing within and between high and low latitude populations grown in a greenhouse common garden. We measured responses to freezing in maternal families of seedlings from two high and one low latitude population.

THE CHAPTERS

Chapter two presents evidence that mild drought increases freezing tolerance of *L. tridentata*. If leaf freezing tolerance is increased during drought while that of xylem is reduced, a mismatch between water supply and demand may occur, decreasing post-freeze performance and survival in the presence of drought. To test this hypothesis we measured survival, leaf loss, plant water potential, gas exchange, cell death, freezing point depression, and leaf specific xylem hydraulic conductance following freeze/thaw events in well-watered and drought greenhouse-grown *L. tridentata* saplings.

Chapter three presents evidence that local adaptation to freezing is present in diploid *Larrea* sp. We compared wood anatomy across three species growing at high and low latitude in the field and saplings from these same field sites grown in a common garden. This comparison offers insights into the ecology and evolution of freezing tolerance in the genus *Larrea*. If freezing limits establishment of warm desert shrubs at high latitudes shrubland distributions may be altered as a result of rising global temperatures, however, local adaptation to freezing may ameliorate low temperature stress. In addition, plastic responses to the environment rather than genetic differentiation may be responsible for differentiation observed across latitudinal gradients. In order to determine if local genetic adaptation to freezing is present in the warm desert genus *Larrea* we investigated wood characters related to freezing tolerance in greenhouse grown saplings and field grown adults from the sapling's site of origin in three diploid species *L. tridentata*, *L. divaricata* and *L. nitida.*

Chapter four presents evidence that past evolution of freezing tolerance has occurred in the genus *Larrea*, and that further evolution of this character is possible in both high and low latitude populations. Freezing tolerance may play a major role in limiting the high latitude expansion of warm desert evergreen shrub populations; however, the extent of this limitation may vary if inter-population variation in freezing tolerance is present. In addition, inter-population variation in freezing tolerance has broad implications for range limitations because it could provide the raw material for natural selection for increased freezing tolerance. Our goal was to test the claim that high latitude populations of the widespread warm desert evergreen species *Larrea tridentata* and *L. divaricata* are locally adapted to freezing and to determine the potential for the further evolution of freezing tolerance in both high and low latitude populations. We investigated responses to freezing in maternal families of 3 month old seedlings from a high and low latitude population of *L. divaricata* from S. America and a high latitude population of *L. tridentata* from N. America. We measured survival, chlorophyll fluorescence and projected plant area before and one week after freezing.

Chapter five summarizes the general findings of this research with special emphasis on the ecological and evolutionary implications.

CHAPTER TWO

THE EFFECTS OF DROUGHT ON THE FREEZING TOLERANCE OF THE LEAVES AND XYLEM OF *LARREA TRIDENTATA*

INTRODUCTION

The well documented effects of drought and freezing stress demonstrate that the physiological tolerance of established individuals is critical for their survival and productivity (Angelopoulos *et al.* 1996; Ball *et al*. 2004; Boorse *et al.* 1998b; Coursolle *et al.* 2000; Drake & Franks 2003; Golding & Johnson 2003). Freezing frequency and long-term minimum temperature may determine the distributional limits of woody evergreen perennials such as *Larrea tridentata* ((Sessé & Moc. ex DC.) Coville) (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002) and *Ceanothus spinosus* (Ewers *et al*. 2003). Across a variety of ecosystems, evergreens frequently experience simultaneous drought and freezing stresses, raising the possibility of interactions between them (Pratt et al. 2005; Mayr et al. 2006a). Unraveling the details of the interaction of drought and freezing stress in desert evergreens may increase our understanding of the limits on plant distribution, particularly at a time when changes in climate may shift the balance between these stresses.

The effects of freezing on water supply may be particularly pertinent for high latitude populations of warm desert evergreens such as *Larrea tridentata* because these populations commonly face water deficits in combination with freezing while maintaining functional leaves. In addition, the freezing temperatures that damage leaf cells may not be the same as those that damage the dead conducting elements found in the xylem (Feild & Brodribb 2001; Cavender-Bares *et al.* 2005) and among evergreens, leaf and xylem freezing responses may be differentially altered by acclimation to low plant water potential. If xylem is more vulnerable to freezing damage than leaves when drought is present this may create an imbalance between water transport capacity and transpiring leaf area.

Cold acclimation can induce a variety of physiological changes, including: reduced growth rate and cellular water content, altered gene regulation, hormone balance and membrane and cell wall properties, and increases in antioxidants (Xin & Browse 2000). Acclimation to cold and drought are similar, with the result that acclimation to one stress confers tolerance of the other and the inhibition of growth and the up-regulation of stress related genes associated with drought confers cold tolerance in many species (Kozlowski & Pallardy 2002; Li *et al.* 2004; Horvath *et al.* 2007; Bouchabke-Coussa *et al.* 2008; Pasquali *et al.* 2008). In many drought-adapted species, cations and sugars accumulate in living cells as Ψ decreases, maintaining cell turgor. This same process can occur as part of cold acclimation (Sakai & Larcher 1987; Walker *et al.* 2008), however, the specifics of such adjustments differ when changes in osmotic potential are induced by drought vs. freezing (Callister *et al.* 2008).

Freeze-thaw events in xylem can compound the effects of drought by reducing leaf specific hydraulic conductance (k_l), placing further limitations on stomatal opening (Cochard & Tyree 1990; Sperry & Sullivan 1992; Tyree & Cochard 1996; Pockman & Sperry 1997; Langan *et al.* 1997). Drought increases the likelihood of freeze-thaw embolism because decreasing plant water potential (Ψ) increases the range of bubble sizes that are sufficient to cause cavitation (Yang & Tyree 1992; Davis *et al.* 1999). The probability that bubbles will lead to vessel embolism depends on conduit volume, and freezing cavitation is closely linked to the vessel size/volume distribution (Sperry & Sullivan 1992; Davis *et al.* 1999; Pitterman & Sperry 2003). For a given vessel size, however, lower Ψ exacerbates freezing cavitation by decreasing the size of the bubble required to cause air-seeding (Davis *et al.* 1999; Ewers *et al.* 2003; Willson & Jackson 2006).

In the living cells of the leaves the primary detrimental effects of freezing are dehydration and mechanical damage to cell membranes during ice formation, both of which may lead to the death of individual cells and/or damage to the photosynthetic apparatus (Pearce 2001). Mechanical damage can cause entire cells to rupture or internal membranes, such as those surrounding the chloroplasts, may be disrupted. Dehydration occurs because the water potential of ice is lower than that of liquid water at the same temperature. Freezing occurs first in the apoplast, followed by a massive re-distribution of water as it flows down a water potential gradient from living cells into the ice-filled apoplast (Ball *et al.* 2004). Plants that have acclimated to drought prior to freezing may be better prepared to tolerate this rapid dehydration than those which have not. Solutes reduce the freezing temperature of the cell sap and allow leaf super-cooling to at least -10^{o}C, while freezing in the apoplast and xylem occurs at temperatures closer to 0^{o}C (Sakai & Larcher 1987). In addition, living cells have often been suggested as agents of xylem embolism repair under tension (Zwieniecki & Holbrook 1998; 2004; Salleo, Trifilo & LoGullo 2006) and a positive effect of drought on the freezing tolerance of living cells could dampen the effects of drought if living cells involved in embolism repair are protected from freezing by the accumulation of solutes.

If leaves withstand freezing during drought better than xylem, a disparity between transpiring leaf area and water transport capacity may result. When transpiration resumes following freezing, reduced leaf specific xylem hydraulic conductance may limit water supply to the leaves, increasing the limits on stomatal opening and thereby reducing carbon gain (Sperry *et al.* 2002). Conversely, if leaves are more vulnerable to lethal damage than xylem, this could effectively protect xylem from catastrophic embolism during subsequent drought by increasing the ratio of absorbing root area to transpiring leaf area. In some species good co-ordination between these traits has been observed (Pratt *et al.* 2005), and frost-sensitive petioles could allow for leaf drop at temperatures too high to cause freeze-thaw xylem embolism (Sakai & Larcher 1987). In contrast, the lack of correlation between leaf and xylem freezing limits in other species demonstrates that these can be independently evolving traits (Cavender-Bares *et al.* 2005; Feild & Brodribb 2001). In addition, living cells have often been suggested as agents of xylem embolism repair under tension (Zwieniecki & Holbrook 1998; 2004; Salleo *et al.* 2006) and a positive affect of drought on the freezing tolerance of living cells could dampen the effects of drought if living cells involved in embolism repair are protected from freezing by the accumulation of solutes.

The relative vulnerabilities of leaves and xylem under freezing and drought may be critical in determining the distribution of aridland species (Ewers *et al.* 2003; Pratt *et al.* 2005). Climate change may alter the existing combinations of drought and freezing such that plant responses are not predicted by single factor manipulations of drought or freezing stress. For example, populations of *L. tridentata* at the far northern edge of the Chihuahuan desert often experience drought and cold in combination during winter months, followed by brief warming periods in which the conditions for gas exchange become favorable. Under these conditions the relative freezing vulnerabilities of leaves and xylem could play a major role in determining plant performance and survival. Although the freezing tolerance of xylem in *Larrea tridentata* is apparently correlated with long-term minimum temperature (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002), the interaction of drought and freezing stress has not been determined for this widespread warm desert evergreen species.

To understand how the interaction of drought and freezing impacts performance and survival of *L. tridentata* we addressed two questions: 1) what are the effects of drought on the freezing tolerance of leaves and xylem in *Larrea tridentata*, and 2) what is the resulting effect on carbon gain and survival following freezing? We compared responses of leaves, xylem and whole plants to freezing under drought and well-watered conditions. We made measurements of survival, leaf loss and re-sprouting, Ψ, gas exchange, relative electrolyte conductivity, freezing point depression, and leaf specific xylem hydraulic conductance on one-year-old green house grown saplings before and after freezing to -8^{o}C, -15^{o}C, -19^{o}C and -24^{o}C. When compared to leaves, we expected xylem to suffer greater freezing damage at low Ψ, because of the effect plant water potential has on the probability of bubble formation within xylem. In contrast, we expected leaves to be less vulnerable when Ψ is low, due to the accumulation of osmotic compounds. Finally, we expected that drought would negatively impact carbon gain and survival following freezing by producing a mismatch between transpiring leaf area and leaf specific xylem hydraulic conductance, but that changes in leaf area could also occur in response to freezing.

MATERIALS AND METHODS

Experimental design

Two separate experiments were performed to test for the effect of drought on performance and survival of one-year old saplings. The first experiment began in October 2007 at which time we measured gas exchange, survival, leaf loss and re-sprouting. The second experiment began in November 2008 at which time we measured leaf specific hydraulic conductance, electrolyte leakage, exotherms, survival, leaf loss and re-sprouting. We measured plant water potential in both 2007 and 2008.

Site description

Our study site was located at the edge of the distribution of *L. tridentata*, near the Five Points area of the Sevilleta LTER (34º 20’N 106º 45’W) at an elevation of 1610 m. Mean annual precipitation, more than 50% of which occurs during the summer monsoon, is 215 mm ranging from 86 to 306 mm, and long-term minimum temperature is -20^{o}C. During the months of December, January and February plants experience an average of 5 nights per month where the temperature reaches -8^{o}C (Moore, 1989-2009).

Seed collection and plant growth

Seed collection and propagation were conducted in the same manner in both years of the study. Seeds were collected in October 2006 and 2007 and germinated in the University of New Mexico research greenhouse where they received only natural light.

Seeds were sown in flats containing a 4:1:1 mixture of sand, peat and perlite, then individual seedlings were transplanted to 1 L containers within 7 days of germination. Seedlings were kept well watered and received spray irrigation once or twice daily depending on temperatures and evaporative demand. One teaspoon of slow release fertilizer (Osmocote 14:14:14) was applied immediately after transplanting and thereafter at four month intervals. Plants were grown in the University of New Mexico greenhouse for 1 year before treatments began. In both 2007 and 2008 experiments began in November and continued through January.

Greenhouse conditions

During plant growth temperatures were maintained between 15^{o}C and 35^{o}C. Mean photosynthetic photon flux density at noon was 1124 μmol m^{-2} s^{-1} with a maximum of 1936 μmol m^{-2} s^{-1} and a minimum of 41 μmol m^{-2} s^{-1}. Humidity was not controlled and mean relative humidity was 27% with a maximum of 72% and a minimum of 8%.

Acclimation conditions

Cold acclimation took place in a ConViron growth chamber (Model #E8 Controlled Environments Limited, Winnipeg, Manitoba, Canada) where conditions were designed to simulate fall weather conditions. Night-time temperature in the growth chamber was 1^{o}C, daytime temperature was 12^{o}C, and light was provided 14 hours per day at an intensity of 1000 μmol m^{-2} s^{-1}. Humidity in the chamber was uncontrolled and showed a similar range as was observed in the greenhouse, between 25-75%.

Watering treatments

In the fall of both 2007 and 2008 we began our experiments with 400 plants which were watered enough to keep soil moist. Well-watered plants were watered using spray irrigation for 5 minutes every day and experienced this same watering regime in both 2007 and 2008. In 2007 drought treatments began 6 weeks before temperature treatments began. Drought plants were watered every other day, resulting in a 50% reduction compared to well-watered plants. Because plant water potential (Ψ) differences generated by this method were small, this regime was altered in 2008. In the second year, watering treatments were begun 6 months before temperature treatments and watering time was gradually reduced to one minute per day over a 3 month period, resulting in an 80% reduction in water availability. Because low temperatures in the acclimation chamber decreased evaporation and transpiration, watering was reduced while plants were in the chamber, with well-watered plants receiving 50 mL of water per day and drought plants receiving 10 mL of water per day. For those plants which were returned to the greenhouse following freezing (gas exchange, survival and leaf loss treatments) plants were watered in the same manner as before acclimation. In 2007 Ψ was measured using a pressure bomb (PMS, Corvalis, Oregon, USA) at midday in the greenhouse and following 7 days of acclimation. In 2008 Ψ was measured at predawn and midday following 7 days of acclimation. *Larrea* leaves lack petioles and green branches break easily in the pressure bomb, so large branches must be cut for successful Ψ measurements. Removal of a branch represented a large proportion of the leaf area and because many of our measurements were proportionate to leaf area, direct measurement of Ψ on treatment plants was impossible. Therefore a separate group of plants were designated for Ψ measurements. In 2007 12 plants were measured in the greenhouse and 9 plants in the acclimation chamber, while in 2008 15 plants were measured in the greenhouse at midday and 5 at predawn.

Temperature treatments

In both 2007 and 2008 freezing methods were identical, but the minimum temperatures varied slightly. During both years plants were randomly assigned to watering and minimum temperature (T_{min}) treatments and each temperature treatment contained between 4-12 samples, depending on the type of measurement made (see the descriptions of individual measurements below for further details on sample sizes). In 2007 measurements were made before and after freezing. In 2008 only post-freeze measurements were made because hydraulic conductance and electrical conductivity measurements are destructive and plants could not be measured before and after freezing. In this case a control treatment was added to improve comparisons across watering treatments and allow determination of changes caused specifically by freezing. 2007 temperature treatments were based on long term climate data from the Sevilleta LTER (Moore 1989-2009) which indicate that minimum temperatures between -9^{o}C and -12^{o}C are typically reached 2-3 times per month during the months of January and February, so we chose to measure plants at one temperature above (-8^{o}C) and one below this range (-15^{o}C). We also chose a temperature just above the long-term minimum temperature for the field site, -19^{o}C. 2007 experiments revealed 1) little to no effect of freezing to -8 in both drought and well-watered plants and 2) survival of drought plants following freezing to -19ºC, therefore, the -8 treatment was removed in 2008 and replaced by a -24ºC. In all cases, freezing took place over the night preceding physiological measurements.

Roots and stems of intact plants rarely experience the same minimum temperatures in nature, so we used a double-chambered freezing apparatus to independently control root and stem temperatures during treatment. Two coolers lined with coiled copper refrigerator tubing were stacked, creating a lower root chamber and an upper canopy chamber. Pots passed through holes in the bottom of the canopy chamber into the lower root chamber. Once pots were in place, holes between the chambers were sealed with strip of high density foam insulation to minimize heat transfer between the lower and upper chambers. A temperature bath (Neslab Model RTE140) filled with water was used to keep root temperature between 1^{o}C and 6^{o}C, while freezing temperatures in the upper chamber were controlled using a second temperature bath (VWR Scientific Products model 1197) filled with a mixture of 60:40 water/ethylene glycol. Using this device, freezing was observed in the soil only in well-watered plants following freezing to -24^{o}C. Treatments started and ended at 10ºC, and cooling/warming proceeded at 1 ºC/min for above zero temperatures and 0.1ºC/min below 0ºC. T_{min} was maintained for 150 mins. Following freezing plants were held at 10ºC until they could be prepared for measurement.

Survival, leaf loss and re-sprouting

In 2007 the proportion of plants surviving was surveyed for 6 weeks following freezing; we monitored 10 plants in each watering x temperature treatment combination. Plants which lost their leaves did so quickly, within 1-2 days after freezing, and lost the entire plant canopy. For this reason leaf loss data is reported as the proportion of plants losing all leaves. Plants which lost all of their leaves but grew new leaves were considered re-sprouts. In 2008 survival and leaf-loss were monitored following freezing to -24^{o}C.

Steady state gas exchange

To determine the effects of freezing on whole plant performance, during 2007 we measured gas exchange using a LiCor 6400 on 10 plants in each watering x temperature treatment combination (LiCor Biosciences, Lincoln, Nebraska, USA). Individual branches were marked with a paint pen, and the same branch was measured before and after freezing. Gas exchange leaf area was measured using Scion Image (Scion Corporation, Frederick, Maryland, USA) to determine number of pixels occupied by leaves, then we used a regression that related number of pixels to cm^2 of leaf area. Inside the LiCor chamber reference CO_2 was set at 400 ppm. To better standardize gas exchange measurements, humidity was controlled more tightly in the LiCor cuvette than during plant growth and was maintained between 30% and 50%. To allow measurement at steady-state, plants were acclimated to light levels in the LiCor chamber for a minimum of 20 minutes before measurements. Light in the chamber was provided by a red-blue lightsource at an intensity of 1655 μmol m^{-2} s^{-1}. Block temperature was set at 20°C for measurements made in the greenhouse. Data were logged every 60 seconds to assess changes in gas exchange before and between measurements. Each observation reported here is a mean of 5 observations recorded by the logger after steady-state was attained.

Freezing point depression in green stems and wood

To determine whether there were differences in super-cooling among samples from drought vs. well-watered plants, freezing point depression was determined by measuring exotherms in green stems and wood in 2008. Exotherms were measured in 12 drought and 12 well watered plants using copper-constantan thermocouple (Model # SMP, Omega Engineering, Inc., Stamford, CT, USA) installed in the middle of each seedling stem, within the outer layer of xylem (Pockman & Sperry 1997). Thermocouples were held in place with tape, and the temperatures of green stems (just below the uppermost leaf junction), the woody bole 1 cm from the soil surface, air and a piece of dry wood were measured every 5 s and averaged every 30 s by a CR-10 datalogger.

Cell death

To quantify electrolyte leakage from cells ruptured during freezing treatments in 2008 we measured electrical conductivity of solutions containing milliQ water (water is treated using a Millipore gradient to an electrical conductivity of < 1 μS/cm) and tissue samples following the methods described in Boorse *et al.* (1998a). We selected 4 drought and 4 well-watered plants for measurement of electrical conductivity of green stems and leaves following three T_{min}, 20°C, -15ºC and -19ºC. Following freeze/thaw treatments, 0.96 g each of fresh stems and leaves were removed from each plant. Only the first 3 ranks of leaves and entirely green stems were used. Stems where wood was apparent were avoided. Green stems were cut into approximately 3 mm segments to ensure that all stem pieces were contained within the water. The sample was divided into 3 - 0.32g subsamples which were each placed into 12 mL of milliQ water in a large test-tube. Samples were covered and kept on a stirring table at room temperature for 24 hours, at which time initial electrical conductivity was measured. Electrical conductivity was measured using a temperature correcting multi meter (WTW model # pH/Cond 340i, Germany) at a reference temperature of 25°C. Variation in room temperature, as measured by the electrical conductivity meter, was low and mean temperature taken at the time of sampling was 21°C. After measurement of initial conductivity, samples were weighed to determine initial weight. They were then autoclaved for 15 minutes (121°C, liquid slow exhaust cycle) to rupture all living cells and obtain maximum conductivity for comparison with the initial measurement. Following cooling, samples were re-weighed and milliQ water was added so that post-autoclave weight was within +/- 0.03g of pre-autoclave weight. Samples were returned to the stirring table and allowed to stir for another 24 hours, at which time maximum conductivity was measured. Relative conductivity was determined as initial/maximum.

Xylem hydraulic conductance

In 2008 we quantified the effects of freezing on plant water transport by measuring xylem hydraulic conductance immediately after freezing for 8 plants in each watering x temperature treatment combination using the Ultra Low Flow Meter, a vacuum chamber method described in Tyree *et al.* (2002). The leaves of *Larrea* are sessile and were removed by hand at the point of attachment along the stem, with terminal meristems cut immediately below the point of attachment using a pair of scissors. The plant was then removed from the soil and the stem was cut under water just below the root/shoot junction, then placed inside a sealed chamber with the cut end attached to Tygon tubing (Saint-Gobain Performance Plastics, Paris, France) containing a solution of 20 mM KCl. Five levels of partial vacuum were applied to the chamber in increments of 2.7 kPa, with the largest pressures applied to plants being 25.3 kPa. Measurements were made with green stems intact, so reported conductance measurements include green stems and wood. Following completion of the freezing cycle plants were kept in the dark at 10ºC until they could be measured. All plants were measured within 8 hours of the completion of the temperature treatment. Hydraulic conductance was determined as the slope of the relationship between the partial pressure in the vacuum flask and the flow of water through the plant stem. Measurements were discarded when the R^2 of this relationship was less than 0.95. Leaf specific hydraulic conductance (k_l) was determined by dividing hydraulic conductance by the total leaf area of the sample. Leaves were removed then dried at 70ºC for 72 hours and weighed. The dry weight was converted to leaf area using a regression created by weighing leaves and scanning them to determine leaf area. Finally, we computed the percent change in k_l

compared to control for comparison with *REC* data. We first calculated a mean control k_l for drought and well-watered plants, then for each observation of k_l we calculated the percent change from mean control k_l for drought or well-watered plants, respectively.

Data analysis

Data analyses were performed in SAS (Ver. 9.2, SAS Institute Inc., Cary, North Carolina, USA). Normality in all data sets was determined using boxplots, stem and leaf plots and normal probability curves and α was set at 0.05 for all ANOVA comparisons. Gas exchange measurements were analyzed using PROC GLM with the REPEATED TIME option for measurements collected before and two weeks after freezing. Watering treatment and minimum temperature were specified in the CLASS statement as the independent variables. Only the plants that survived freezing with leaves were included in the analysis, which removed all but one of the -19^{o}C plants from the time series analysis of gas exchange. Water potential data were analyzed in EXCEL using a paired t-test. ANOVA statistics for leaf and green stem electrical conductivity, exotherms, and leaf specific hydraulic conductance were generated using proc GLM with water and temperature treatments as the independent variables. Pairwise comparisons were generated using the LSMEANS statement in proc GLM. Only planned comparisons were used and α was set at 0.05 for all pairwise comparisons. Hydraulic conductance data were log transformed for analysis to improve normality in the data. Survival and leaf loss data were analyzed by temperature treatment using PROC FREQ with water as the independent variable. Separate pairwise Chi-Square tests were performed to check for difference between temperature treatments. We used a sequential Bonferroni correction to determine significance for all pairwise comparisons of survival and leaf loss data (Rice 1989).

RESULTS

Plant water potential

In 2007 watering treatments were sufficient to cause a significant difference in plant water potential (Ψ) in the greenhouse ($P = 0.0003$). Mean Ψ for control plants was -2.7 *MPa*, and -3.8 *MPa* for drought plants. Differences were not significant following one week in the acclimation chamber ($P = 0.4806$), however, where mean Ψ for the high water treatment was -2.4 *MPa* for control plants and -2.8 *MPa* for drought plants. In 2008, there were significant differences in Ψ following one week in the acclimation chamber, well-watered plants had a mean Ψ of -1.8 *MPa* and drought plants had a mean Ψ of -3.5 *MPa* ($P < 0.0001$).

Freezing point depression, exotherms

Mean exotherm temperature was significantly in an ANOVA comparing well-watered plants (-7.5^{o}C) and drought plants (-9.2^{o}C; $F = 6.03$, *df* =1, $P = 0.0208$). In both well-watered and drought treatments there was a trend for exotherms in green stems to occur at lower temperatures than woody tissues, but these differences were not significant in an ANOVA (F = 2.15, *df* =1, P = 0.1544), resulting in no significant interaction between watering treatment and tissue type (F = 0.05, *df* = 1, P = 0.8250).

Survival, leaf retention and re-sprouting

In 2007, before freezing, 1/3 of drought plants died following the application of watering treatments and there were significant differences in the proportion of drought and well-watered plants surviving the application of watering treatments (*Table 2-1*). There were no differences between drought and well-watered plants in survival or re-sprouting following freezing to -8°C, all plants survived this temperature with their leaves intact. Following freezing to -24°C all drought and well-watered plants died. Between these extremes, there were differences between the response of the two watering treatments when plants were frozen to -15°C and -19°C, a range in which drought plants were much more likely than well-watered plants to survive (*Figure 2-1*) and retain their leaves (*Figure 2-2*). We found significant differences between drought and well-watered plants in proportion of plants retaining leaves and proportion of plant re-sprouting following freezing to -15ºC but no differences in survival (*Table 2-1*). There were no significant differences between watering treatments in survival following freezing to -19°C, however (*Table 2-1*).

Figure 2-1. Survival of drought and well-watered plants before and after freezing. *Survival was measured following 5 minimum temperature treatments (T_{min}). Some drought plants died before freezing following the application of watering treatments (T_{min} 20°C). All drought and well-watered plants survived freezing to -8°C, but more drought than well-watered plants survived freezing to -15°C and -19°C. Zeros indicate that no plants survived freezing to -24°C.*

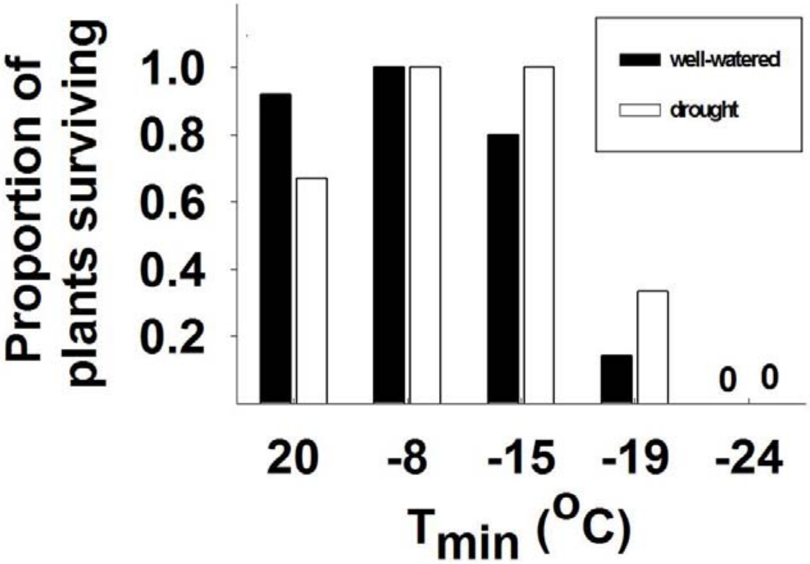


Figure 2-2. Leaf retention in drought and well-watered plants before and after freezing.

The proportion of plants retaining leaves was measured following 5 minimum temperature treatments (T_{min}). Some drought plants lost all their leaves before freezing following the application of watering treatments (T_{min} 20^{o}C). Plants that lost their leaves following the drought treatment were not used in further low temperature experiments. All drought and well-watered plants retained their leaves freezing to -8^{o}C, but more drought than well-watered plants retained their leaves following freezing to -15^{o}C and -19^{o}C. Zeros indicate that no well-watered plants retained their leaves following freezing to -19^{o}c and neither drought nor well-watered retained their leaves following freezing to -24^{o}C.

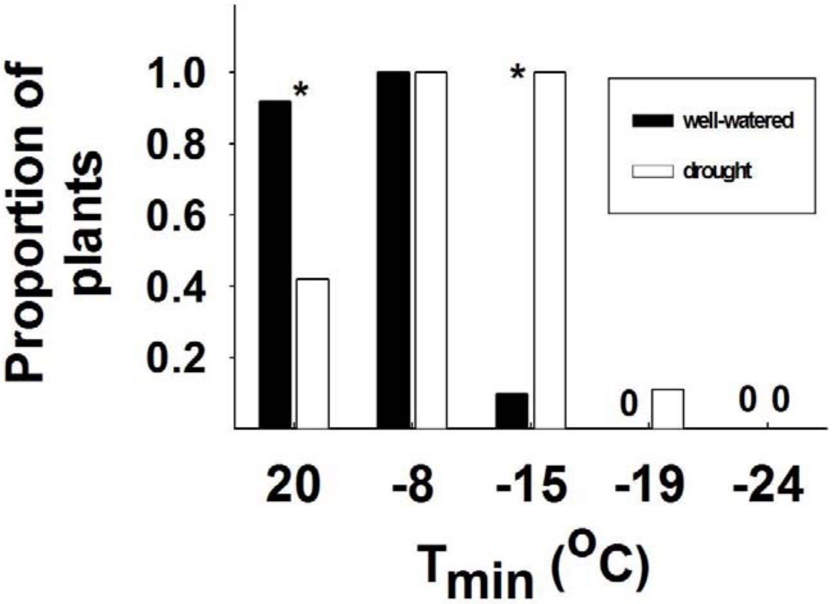


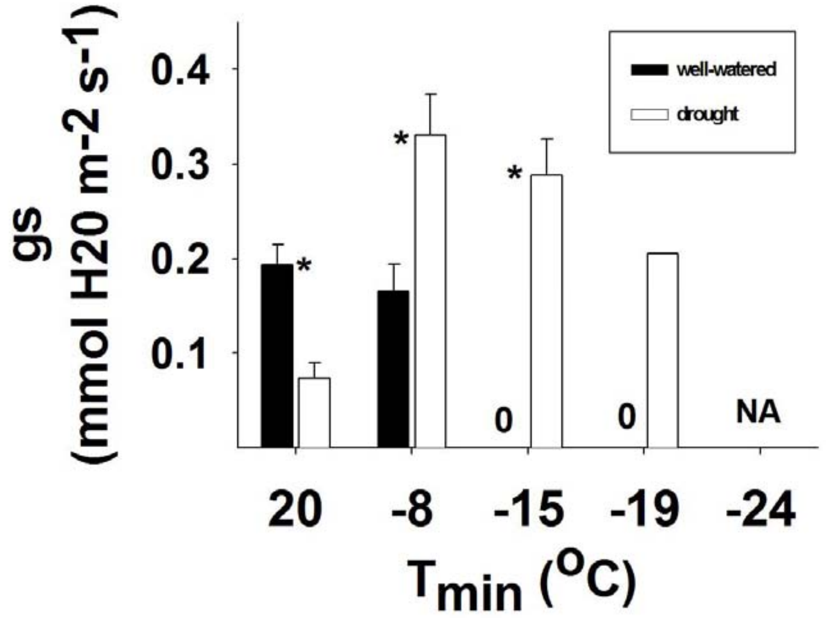
Table 2-1. Results of a Chi-square test for the effects of watering treatment on the proportion of plants surviving, loosing leaves and re-sprouting.
** represents significance at $\alpha = 0.05$; ** at $\alpha = 0.01$; *** at $\alpha = 0.001$; **** at $\alpha = <0.0001$.*

Minimum temperature	***Dependant variable***	***df***	**X^2**
20^oC	*Survived*	1	**19.17******
	Survived with Leaves	1	**56.54******
	Re-sprouting	1	**28.57******
-15^oC	*Survived*	1	1.15
	Survived with Leaves	1	**11.25*****
	Re-sprouting	1	**6.56***
-19^oC	*Survived*	1	1.17
	Survived with Leaves	1	3.41
	Re-sprouting	1	0.05

Gas Exchange

Before freezing, plants from the drought treatment exhibited roughly 50% lower stomatal conductance (g_s) than well-watered plants *(Figure 2-3)* and correspondingly lower rates of assimilation and transpiration (*data not shown*). Immediately after freezing both drought and well-watered plants had near-zero rates of gas exchange and there was little difference between the two watering treatments (*data not shown*). However, two weeks after treatment, drought plants exhibited higher g_s than well-watered plants across all four T_{min} treatments. In the -15^oC and -19^oC treatments, gas exchange could not be measured in well-watered plants because they had lost all leaves and had not yet re-sprouted at two weeks. Small leaf areas in re-sprouted well-watered plants resulted in high gas exchange rates, which are calculated on a leaf area basis. The patterns for photosynthesis and transpiration were similar to those described for g_s, with drought plants demonstrating lower rates than well-watered plants before freezing but higher rates two weeks following freezing (*data not shown*). A time series MANOVA revealed a significant effect of time (F = 62.48, *df* = 6, P < 0.0001), time by watering treatment (F = 12.73, *df* = 6, P = 0.0027), time by T_{min} (F = 9.53, *df* = 6, P = 0.0059) on photosynthesis, transpiration and stomatal conductance; but there was not a significant three-way interaction (F = 2.12, *df* = 6, P = 0.1835).

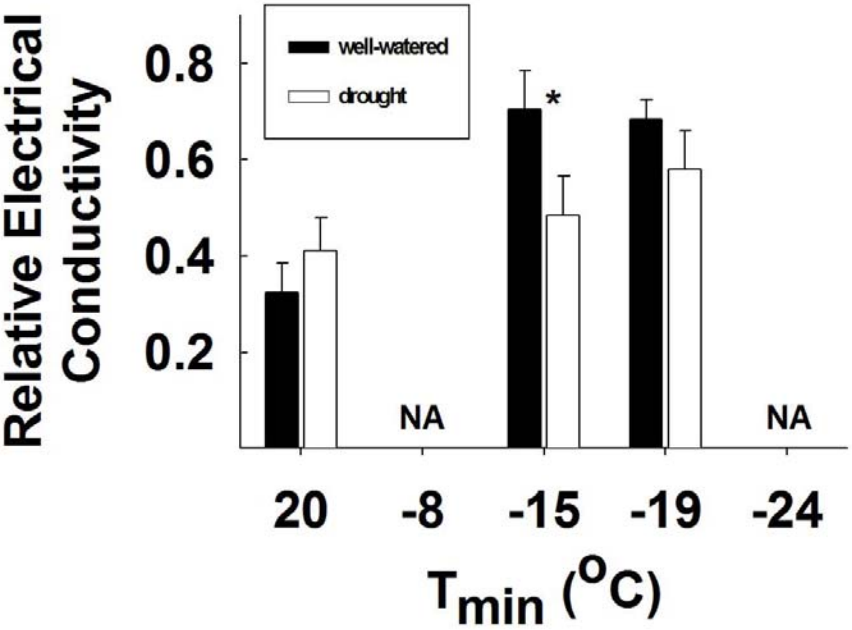
Figure 2-3. Stomatal conductance in drought and well-watered plants before and two weeks after freezing.
*Gas exchange was measured following five minimum temperature treatments (T_{min}). Before freezing (T_{min} 20°C) mean stomatal conductance (g_s) of drought plants was significantly lower than that of well-watered plants, but two weeks following freezing to -8°C, -15°C and -19°C drought plants had significantly higher mean g_s than well-watered plants. Error bars represent one standard error and * represents significant differences in LSMEANS pairwise comparisons. Placement of a zero instead of a bar indicates cases where plants died and could not be measured, while placement of NA indicates that measurements were not made at that temperature. Data from are from 2007, except for data in the -24°C treatment, which were collected in 2008.*



Cell death

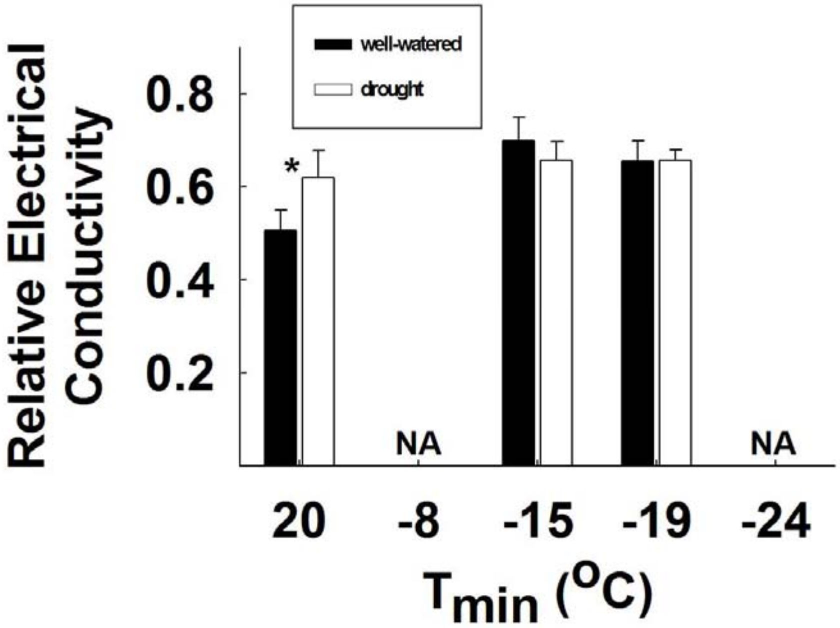
Drought plants had higher relative electrolyte leakage (*REC*) before freezing than well-watered plants, but freezing increased *REC* in well-watered plants more than in drought plants. ANOVA test revealed that water alone was not a significant predictor of *REC* (*Table 2-2*), thought there were significant differences across T_{min} and the interaction of water and temperature. Leaves (*Figure 2-4*) showed a more drastic increase in *REC* following freezing than green stems (*Figure 2-5*), and though there was a trend for green stems of drought plants to have lower *REC* than well-watered plants following freezing to $-15^{o}C$ there were no significant differences. Pairwise comparisons revealed significant differences between leaves of well-watered and drought plants in electrolyte leakage following freezing to $-15^{o}C$.

Figure 2-4. Relative electrical conductivity of leaves before and after freezing. *Relative electrical conductivity (REC) was measured at five minimum temperatures (T_{min}). Before freezing (T_{min} 20°C) drought plants had higher mean REC than well-watered plants, but following freezing to -15°C drought plants had significantly lower mean REC than well-watered plants. There was also a trend for drought plants to have lower mean REC than well-watered plants following freezing to -19°C. Error bars represent one standard error and * represents significant differences in LSMEANS pairwise comparisons. Placement of a zero instead of a bar indicates cases where plants died and could not be measured, while placement of NA indicates that measurements were not made at that temperature.*



Green stems had higher *REC* than leaves overall and there was a significant difference between the two tissue types. In both drought and well-watered plants *REC* was higher before freezing in green stems than in the leaves, for which *REC* increased more dramatically following freezing, and there was a significant effect of tissue type x temperature (*Table 2-2*). In green stems, increases in *REC* following freezing for drought plants were small compared to that observed in well watered plants (*Figure 2-5*), for which *REC* increased dramatically as a result of freezing. In leaves *REC* increased sharply following freezing in both well-watered and drought plants, although the change was greater for well-watered plants compared to drought plants, and there was a significant interaction between watering treatment and tissue type (*Table 2-2*).

Figure 2-5. Relative electrical conductivity of green stems before and after freezing. *Relative electrical conductivity (REC) was measured at five minimum temperatures (Tmin). Before freezing (Tmin 20^{o}C) drought plants had higher mean REC than well-watered plants, but following freezing there was a trend for drought plants to have equal or lower mean REC than well-watered plants. Error bars represent one standard error and * represents significant differences in LSMEANS pairwise comparisons. Placement of NA indicates that measurements were not made at that temperature.*



Xylem hydraulic conductance

Before freezing drought plants had lower k_l than well-watered plants, but the reverse was true following freezing *(Figure 2-6)*. The differences between drought and well-watered plants were significant in a two-way ANOVA (*Table 2-2*) and there was a significant interaction between water and temperature. No plants survived freezing to -24°C but drought plants still exhibited water transport capacity, although at severely reduced rates. We observed a trend of decreasing k_l in drought plants as T_{min} decreased, and there was a significant effect of T_{min} in the ANOVA model. However, this difference was driven by differences in k_l between control and T_{min} treatments, not by differences between the freezing treatments, and pairwise comparisons of the temperature treatments revealed no significant differences in k_l between -15°C, -19°C and -24°C temperature treatments.

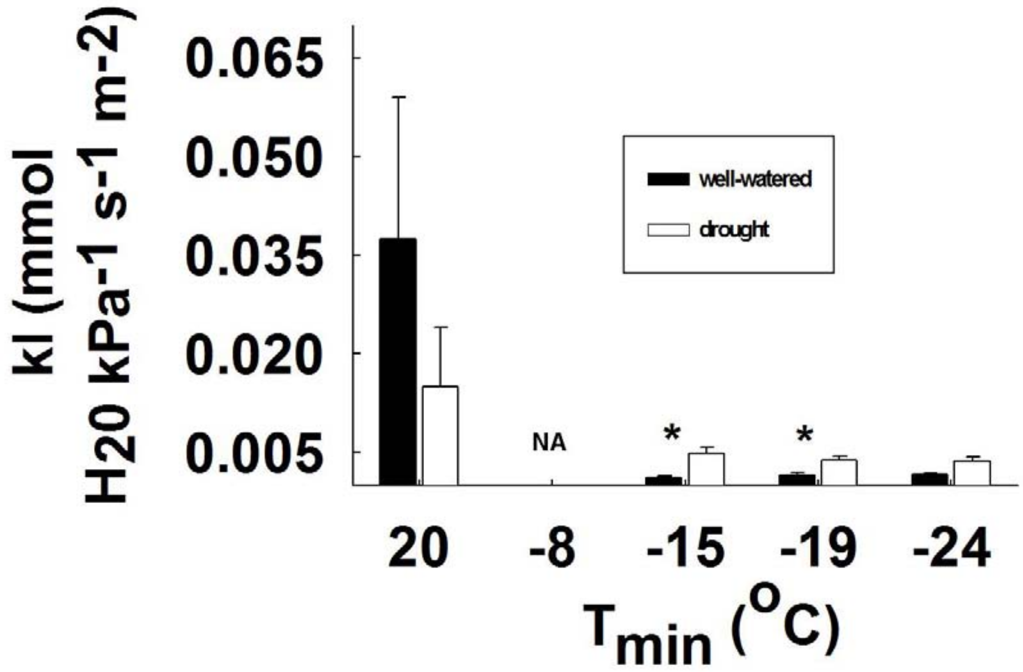
Table 2-2. Results of an ANOVA testing for the effects of watering treatment, temperature treatment, tissue type on leaf specific hydraulic conductance and relative electrical conductivity of high latitude L. tridentata.

*Because of unequal sample sizes Type III sum of squares were used to determine significance. * represents significance at $\alpha = 0.05$; ** at $\alpha = 0.01$; *** at $\alpha = 0.001$; **** at $\alpha = <0.0001$.*

	df	***Relative electrical conductivity***	***% Loss leaf specific hydraulic conductance***	***df***	***Leaf specific hydraulic conductance***
Watering treatment	1	1.72	**130.46******	1	**11.24****
Temperature treatment	2	**35.49******	0.87	3	**33.21******
Tissue	1	**29.92******	**50.87******	1	**46.75******
Watering treatment x Temperature treatment	2	**13.62******	3.00	3	**4.88****
Watering treatment x Tissue	1	**8.16****	**29.23******	1	0.44
Temperature treatment x Tissue	2	**7.67*****	0.92	3	**4.26****

Figure 2-6. Leaf specific hydraulic conductance in drought and well-watered plants following before and after freezing.

*Leaf specific hydraulic conductance (kl) was measured at five minimum temperatures (T_{min}). Before freezing (Tmin 20°C) drought plants had lower mean kl than well-watered plants, but following freezing to -15°C and -19°C drought plants had significantly higher mean k_l than well-watered plants. There was also a trend for drought plants to have higher mean k_l than well-watered plants following freezing to -24°C. Error bars represent one standard error and * represents significant differences in LSMEANS pairwise comparisons. Placement of a zero instead of a bar indicates cases where plants died and could not be measured, while placement of NA indicates that measurements were not made at that temperature.*

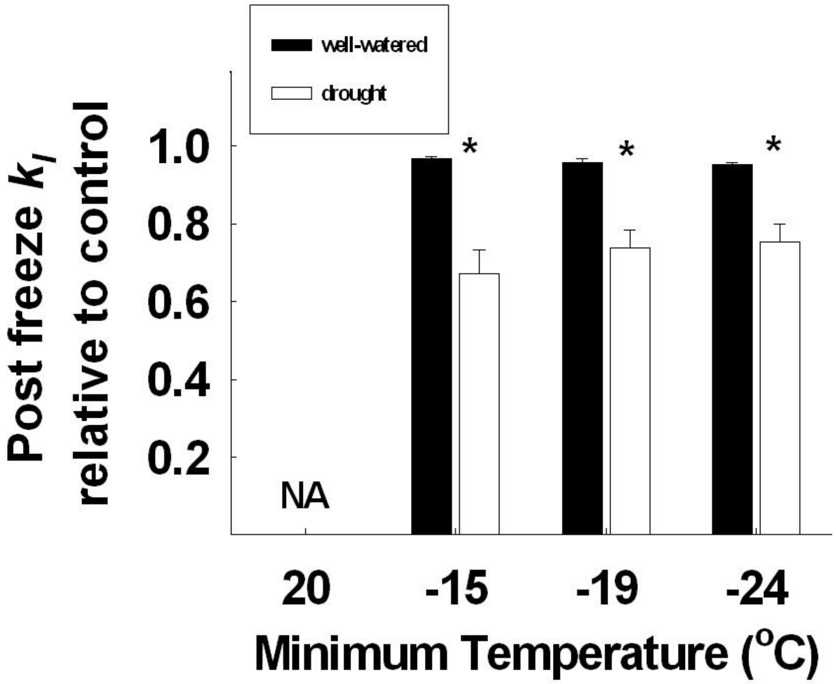


Relative balance between leaf and xylem vulnerability

There were differences between the watering treatments in the balance between leaf and xylem function following freezing and a two-way ANOVA revealed significant differences in percentage of xylem function lost following freezing. Compared to well-watered plants, drought plants exhibited a smaller percent change in k_l relative to control (*Table 2-2; Figure 2-7*). Following freezing to -15^{o}C well-watered plants experienced nearly 100% loss of xylem function compared to control, accompanied by a 70-80% increase in leaf cell death (*Figure 2-4*). In contrast, reductions in k_l and leaf *REC* of drought plants were more closely matched at 70-80% for xylem (*Figure 2-7*) and 50-60% for leaves (*Figure 2-4*). In addition, leaf damage at -15^{o}C was less severe than xylem embolism in drought plants, while well-watered plants exhibited similar levels of leaf and xylem damage following freezing to -15^{o}C.

Figure 2-7. Percent loss of leaf specific hydraulic conductance in drought and well-watered plants following freezing.

*The percent loss of leaf specific hydraulic conductance (k_l) was calculated for each plant relative to mean pre-freeze k_l. The mean percent loss of k_l was lower for drought plants than for well-watered plants for all minimum temperature treatments. Error bars represent one standard error and * represents significant differences in LSMEANS pairwise comparisons. Placement of NA indicates that measurements were not made at that temperature.*



DISCUSSION

In evergreen species the relative vulnerabilities of leaves and xylem to combined drought and freezing stress may have major consequences for plant performance and survival. Acclimation to drought induces solute accumulation in leaves, thereby protecting them from freezing, while at the same time decreasing xylem water potential increases the likely-hood of freeze-thaw embolism, therefore we expected that the balance between water supply and demand following freezing would be disrupted under drought conditions. Our data show that freezing to relatively high sub-zero temperatures (e.g. -8°C) produces similar effects regardless of plant water potential, and these temperatures occur on a regular basis at our field site. Likewise, our severe freezing treatment (e.g. -24°C) caused 100% mortality regardless of water status. Contrary to our predictions, however, drought improved plant performance and survival following freezing to intermediate temperatures of -15°C and -19°C. By -19°C, close to the long-term minimum temperature for this region, the greater freezing tolerance of leaves in drought plants was no longer evident, but a few drought plants survived with leaves intact and reduced, but apparently sufficient water transport capacity. These data indicate that this species can survive freezing temperatures near the long-term minimum temperature recorded at the Sevilleta LTER, but only in the presence of drought.

In both 2007 and 2008 the drought imposed by our watering treatment was sufficient to induce changes in plant function and performance. Although the magnitude of the drought we imposed was larger in 2008 than in 2007, we are still able to draw some general conclusions about the effect of drought on freezing tolerance. In both years, drought plants reduced growth and lost leaf area in response to the drought treatments, an indication that physiological changes related to drought were taking place. The plant water potentials we observed here are higher than those generally observed in *L. tridentata* in the field. However, plant water potentials in our drought treatment are similar to the typically higher values that occur in plants near Five Points during winter (Martinez-Vilalta & Pockman 2002).

We expected xylem to experience greater freezing damage at low plant water potential, compounding pre-existing differences between drought and well-watered plants. Although drought plants had lower rates of stomatal conductance and lower leaf specific hydraulic conductance than well-watered plants before freezing, this trend was reversed after freezing. In fact, drought plants performed better than well-watered plants following freezing across all the different physiological measurements and this resulted in greater post-freeze performance and survival. This result may be due in part to the fact that drought significantly increased super-cooling in both green stems and wood. However super-cooling in drought plants only delayed the occurrence of freezing exotherms from -7^{o}C in well-watered plants to -9^{o}C in drought plants, suggesting that the differences between the watering treatments in the -15^{o}C and -19^{o}C treatments were due to differences in plant physical characteristics or responses.

The fact that leaves were more tolerant of freezing in the presence of drought was not surprising, and similar results have been reported in other woody evergreens (Cavender-Bares *et al.* 2005; Ewers *et al.* 2003). Greater whole plant performance of drought vs. well-watered plants following freezing to -15^{o}C may have been the result of a better balance between leaf and xylem vulnerability in drought plants. In contrast, other studies have found no relationship between leaf and xylem freezing tolerance in vessel-less angiosperms (Feild & Brodribb 2001) or conifers (Coopman *et al.* 2008). However, across species of oaks, longer-lived leaves have been shown to have higher resistance to freezing and a stronger correlation between leaf and stem traits (Cavender-Bares *et al.* 2005), so such coordination may be expected in evergreen leaves like those of *L. tridentata*.

Our observations of k_l in drought plants are similar to those of Pockman & Sperry (1997) and Martinez-Vilalta & Pockman (2002) in which field collected *L. tridentata* became completely embolized between -16^{o}C and -20^{o}C. As in our study, plant water potential in these studies ranged from -3 to -4 *MPa* at the time of freezing. Unexpectedly, we saw that drought decreased the vulnerability of xylem to freeze-thaw embolism. Our measure of water transport capacity was scaled to the total amount of leaf area before freezing, which means that leaf loss following drought could have contributed to higher leaf specific hydraulic conductance we observed. In our experiment, differences between drought and well-watered plants were much more apparent in our measure of leaf damage (cell death) than in our measure of xylem damage (loss of conductance). Leaf damage was apparent immediately following freezing, and plants that were damaged lost all, not a portion of their leaves in response to freezing, indicating that a mismatch between water transport and water use was not to blame for the death of leaves. Finally, greater leaf loss in well-watered vs. drought plants at -15^{o}C in concert with a much higher rate of leaf loss in well-watered plants at that temperature suggest that dehydration and mechanical cell damage is likely a primary cause for the leaf drop we observed.

In other experiments measuring the effects of drought and freezing on xylem embolism drought significantly increased xylem vulnerability (Davis *et al.* 1999; Ewers *et al.* 2003; Willson & Jackson 2006). What differences could account for the opposite effect we observed? One difference may be that, while previous studies have measured the combined effects of drought and freezing on xylem embolism of branches that were droughted and/or frozen following their removal from the plant, in our study plants were droughted and frozen intact. The preservation of whole-plant function by this method could have maintained the ability of living cells to respond to freezing by further mobilizing a stress response or the up-regulation of genes. Although leaf specific hydraulic conductance was drastically reduced following freezing to -15°C in both watering treatments, drought plants maintained water transport capacity above a critical threshold that allowed them to retain their leaves and resume carbon gain following freezing. The ability to maintain a minimum leaf specific hydraulic conductance under the prevailing combination of drought and freezing is likely a key factor in maintaining an evergreen habit in the presence of these combined stresses (Pratt *et al.* 2005).

Loss of leaf area could effectively protect xylem from further damage during extended sub-lethal cold temperatures by eliminating dehydration and transpiration before new xylem is initiated in the spring. We observed for re-sprouting in drought plants following freezing to -19°C that could be extremely important for survival of *L. tridentata* populations existing at or near the thermal limits of the species. Boorse *et al.* (1998b) and Ewers *et al.* (2003) both report significant re-sprouting in more cold tolerant species of *Ceanothus*, and re-sprouting following freezing may be a cost-effective way of tolerating extreme, but infrequent, freezes.

When freezes occur more frequently, refilling may occur over short time scales under negative xylem pressure (Nardini *et al.* 2008; Zwieniecki & Holbrook 1998). In our case, refilling would have taken place during the time when plants were thawing and waiting to be measured. Plants spent between one and six hours at 10°C in the chamber before hydraulic conductance measurements were made, but a post-hoc ANOVA showed no significant effect of the time a plant waited between freezing and measurement on leaf specific hydraulic conductance for any of the watering treatments or tissue types. The time course of vessel embolism and refilling can be very short, even within minutes in leaves (Trifilo *et al.* 2003) to a few hours in xylem (Bucci *et al.* 2003; Holbrook *et al.* 2001) though some have reported that it takes place over a few days (Cordero & Nilsen 2002). Mayr *et al.* (2006b) demonstrated that needles and twigs of conifers can freeze and thaw more than 100 times during a season. High latitude populations of Larrea are likely to experience a similarly large number of freeze thaw cycles per year, making refilling an important phenomenon.

In addition to higher leaf specific hydraulic conductance following freezing, there was also a trend for embolism to increase with decreasing minimum temperature among drought plants, a pattern previously observed in *L. tridentata* (Martinez-Vilalta & Pockman 2002). One possible explanation for this is that mortality among the xylem parenchyma cells that may support refilling increases as minimum temperature decreases progressively. Our survival data also support this conclusion, and other studies have implicated the death of living cells as the cause of increasing loss of hydraulic conductance with decreasing minimum temperature (Ball *et al.* 2006; Cavender-Barres *et al.* 2005).

An alternative explanation is that biophysical changes in drought plants prevented, rather than repaired, freezing embolism. Both living and non-living cells might have benefitted from drought through increased tolerance for changes in water content associated with freezing, as was observed in the cold acclimated leaves of *Eucalyptus pauciflora* (Ball *et al.* 2004). There is evidence that physical properties of cell walls can change when plants are grown in the presence of stress (Xin & Browse

2000). In fact, the main protective effect of acclimation to freezing tolerance is not an increase super-cooling but higher tolerance of the mechanical deformations of cell walls caused by ice crystal growth (Roden *et al.* 2009). Such mechanical stress affects not only living cells, but dead xylem vessels as well, as it may disrupt the tight connections between adjacent cells, eliminating the possibility of water re-distribution (Ball *et al.* 2004).

Studies on a wide variety of species have shown that gene regulation (Bouchabke-Coussa *et al.* 2008; Pasquali *et al.* 2008), accumulation and redistribution of osmolytes (Ameglio *et al.* 2004), alterations in hormone expression (Pagter *et al.* 2008) and biophysical changes in cell membranes that occur during freezing acclimation also occur during drought; such changes could convey increased freezing tolerance (Pearce 2001; Horvath *et al.* 2007; Peng *et al.* 2008). In addition, changes in ionic concentrations in xylem sap, which are mediated by living cells, has a positive effect on xylem hydraulic conductance in a variety of species in the field (Trifilo *et al.* 2008). For both leaves and green stems we saw that drought plants had higher electrolyte leakage than well-watered plants, but lower electrolyte leakage following freezing. These data suggest that some osmotic adjustment took place in drought plants, and others have suggested that such adjustment is congruent with a model of embolism refilling that includes living cells (Ameglio *et al.* 2004). Bucci *et al.* (2003) found that petiole sugar content was mediated by living cells and was highest during the period when embolisms were refilled. Measurement of this trait in the green stems of *Larrea tridentata* could help distinguish between these two possibilities. Other studies have demonstrated a role for phloem in rapid refilling of embolism (Salleo *et al.* 1996; 2004; Salleo *et al.* 2006; Zwieniecki *et al.*

2004) and techniques that interrupt the phloem could further distinguish between physical and biological causes.

CHAPTER THREE

VARIATION IN WOOD CHARACTERISTICS RELATED TO FREEZING TOLERANCE IN FIELD AND GREENHOUSE POPULATIONS OF DIPLOID *LARREA* SP. (ZYGOPHYLLACEAE) FROM HIGH AND LOW LATITUDES IN NORTH AND SOUTH AMERICA

INTRODUCTION

It is well known that water availability limits establishment and survival in warm deserts, however, freezing can also occur in deserts and freezing tolerance may be especially important for the survival of woody evergreen perennials that remain active through the cold season. If climate change decreases either the intensity or the frequency of freezing conditions experienced by high latitude warm desert evergreen populations, species distribution may shift as plants are released from freezing stress, while increases in freezing frequency and severity may cause range shrinkage at high latitudes. Plastic responses and/or local adaptation to temperature regime, on the other hand, may ameliorate the effects of climate change on plant performance and survival. Many studies suggest that plants vary in freezing vulnerability across latitudinal and altitudinal gradients, and this pattern has been observed in species as diverse as oaks (Tyree & Cochard 1996; Nardini *et al.* 2000), Rhododendrons (Cordero & Nilsen 2002), Tasmanian tree species (Feild & Brodribb 2001), chaparral shrubs (Ewers *et al.* 2003), and Asiatic species of the genus *Cornus* (Noshiro & Baas 2000). Though a strong genetic component for freezing tolerance has been reported in diverse species such as *Arabidopsis thaliana* (Zhen & Ungerer 2007), *Pseudotsuga menziesii* (St. Clair 2006), and *Dodonaea viscosa* (Liu & Noshiro 2003), a significant contribution of plastic responses of wood characters to environment has been detected in a wide variety of species, including: tropical tree species *Metrosideros polymorpha* (Fisher *et al.* 2007), *Eucalyptus globulus* (Leal *et al*. 2003) and *Eucalyptus grandis* (Thomas *et al.* 2007), Fynbos species *Protea repens* and *Cunonia capensis* (February & Manders 1999), evergreen aridland species *Buddleja cordata* (Aguilar-Rodriguez *et al*. 2006), and cacti (Stevenson & Mauseth 2004).

The major physiological effects of freezing include disruption of cellular membranes caused by intercellular ice formation (Pearce 2001; Roden *et al.* 2009) and the interruption of water transport in the xylem (Sakai & Larcher 1987). Air is less soluble at low temperatures so air bubbles can form in vessel and tracheary elements during freezing. The probability that bubbles will lead to vessel embolism depends on vessel volume and xylem water potential. Conduit volume is an important determinant of freezing cavitation because larger bubbles can form in larger conduits, and these larger bubbles expand at higher xylem water potential (Sperry *et al.* 1994; Langan *et al.* 1997; Pitterman & Sperry 2003). Thus, for a vessel of a given size, decreasing water potential increases the likelihood of freezing cavitation by decreasing the size of bubble required to cause cavitation, concomitantly reducing the critical vessel size required to avoid embolism (Davis *et al.* 1999). As a result, the interaction between water potential and temperature could be particularly important in warm deserts where drought is omnipresent. Predicted changes in xylem under simultaneous freezing and drought stress include decreased investment in efficient, large volume vessels which are more prone to freezing cavitation, and increased redundancy of smaller, safer vessels (Carlquist and

Debuhr 1977). This pattern has been reported across 31 species of the genus *Symplocos* (van den Oever *et al.* 1981) and across angiosperm and gymnosperms species from a wide variety of environments (Sperry *et al.* 2008).

Species of the genus *Larrea* were chosen for this study because they are members of what may be the most widespread warm desert shrub genus in the Americas (Ezcurra *et al.* 1991; Henrickson & Johnston 1997), spanning broad latitudinal ranges from 34^{o}N to 20^{o}N in North America and from 46^{o}S to 22^{o}S in South America (Barbour 1969). Across this latitudinal range on each continent, freezing is common at high latitudes but rare in low latitude populations, providing a unique opportunity to study the effects of latitude on freezing tolerance in a warm desert evergreen.

Previous work has demonstrated latitudinal variation in wood characters related to freezing tolerance in *L. tridentata* (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002). However, these studies compared *L. tridentata* from low latitude Sonoran Desert populations, which may be tetraploid, with high latitude populations from the Chihuahuan Desert, which are diploid (Duran *et al*. 2005). Polyploidy is well known to impact cell size, with larger genomes resulting in larger cells (e.g. Stupar *et al.* 2007). A subsequent comparison of vessel sizes in *L. tridentata* designed to account for differences both in ploidy and climate found that ploidy, rather than environmental causes, determined the largest proportion of the observed variation in vessel size across the Sonoran, Mojave and Chihuahuan deserts (A.P. Tyler Master's Thesis 2004). In addition, previous studies have not addressed the possibility that observed differentiation is the result of plastic responses to the growth environment as opposed to genetic local adaptation.

The findings of previous work prompted the current study in which we compare wood characters among field and greenhouse grown diploid species of *Larrea* across their entire ranges. Of the four species present in South America, *L. divaricata* is most similar in physiology, morphology and ecology to its North American cousin *L. tridentata*; both are diploid and occupy similar ecological niches (Barbour 1969). Populations of similar ploidy are likely to have similar constraints on cell size, and therefore observations of differences in vessel sizes are more robustly attributed to the increased severity and occurrence of freezing. We also compared these two species to another diploid *Larrea* species, *L. nitida*, which occurs in the coldest habitats occupied by this genus in South America (Ezcurra *et al.* 1991) and therefore might be expected to exhibit the most striking adaptations to freezing.

To determine if local adaptation of wood characters related to freezing exists in the warm desert genus *Larrea* in the absence of differences of ploidy, we collected seeds and wood samples at 2 low and 3 high latitude field sites in the Chihuahuan (North America) and Monte Deserts (South America). We measured vessel size distributions of field collected stems and of saplings grown in a greenhouse common garden. We asked the following questions: 1) Do wood characters related to freezing tolerance vary according to latitude of origin across the natural range of *L. tridentata* and *L. divaricata*? 2) How do wood characters in these species compare with *L. nitida*, a species occurring in colder habitats, and 3) Do wood characters related to freezing have a plastic component in this genus? If xylem vessel diameter is plastic or changes over development, we expected that we would find differences among adults across latitude and but not among saplings grown in a common garden. On the other hand, if local adaptation to freezing has occurred in these species, we expected to observe differences among adults across latitude and among saplings from a common greenhouse environment.

MATERIALS AND METHODS

Sampling design and field sites

We compared wood characters of one-year-old saplings grown in a common garden with wood from field populations. Since saplings of *Larrea* were not encountered in the field, field samples of branches 1 cm in diameter or less were collected from 8 adult plants at each field site. Wood samples of *L. nitida* were collected at the high latitude South American site in 2004. Seed and wood samples of *L. tridentata* were collected at the high and low latitude North American sites in the winter of 2006. The same collections were made for high and low latitude populations of *L. divaricata* in South America in the winter of 2008. Wood samples were wrapped in plastic to reduce dehydration during transport and frozen in the lab until they could be sectioned.

The high latitude North American site is located at the edge of the distribution of *L. tridentata*, near the Five Points area of the Sevilleta Long Term Ecological Research site (34º 20’N 106º 45’W) at an elevation of 1610 m. Mean annual precipitation, more than 50% during the summer monsoon, is 215 mm and ranges from 86 to 306 mm. Long-term minimum temperature is between -14ºC and -20ºC, while mean annual temperature is 9ºC (Moore 1989-2009).

The low latitude North American site is located near Higurillas in Querétaro, Mexico (20^o 54’N 99^o 45’W) at an elevation of 1580 m. This site is within the western rain shadow of the Sierra Madre Oriental in the Central Mexican Plateau, one of the driest regions in Mexico. Altitudes range from 1300 to 2000 m. Mean annual precipitation range between 380 and 400 mm. Long-term minimum temperature in December, January and February during the period from 1982-1998 was 14^{o}C and mean annual temperature was between 18^{o}C and 22^{o}C. The rainy season occurs between June and December with a peak in September (Horacio Paz, *personal communication*).

The low latitude South American site is located near Chamical, La Rioja, Argentina, at the Northern end of the Monte Desert (29^{o}25’ S 66^{o}52’ W) at an elevation of 517 m. The mean annual temperature (1931 -2005) is 20^{o}C (www.globalbioclimatics.org). Mean annual precipitation is 100-200 mm depending on slope and aspect. Mountain valleys experience only a handful of nights each month below zero in June, July and early August with a long-term minimum temperature of -7^{o}C or -8^{o}C. (P. Searles *personal communication*).

The high latitude South American site is located near Bajada del Diablo, Chubut, Argentina at the far southern end of the Monte Desert (42^{o}31’ S 68^{o}15’ W). Very little climate data is available for the region, however, the climate is cold and arid with the majority of precipitation falling in the winter (Ezcurra *et al.* 1991). At nearby Maquinchao mean annual temperature is 9.4^{o}C and mean annual precipitation is 183 mm.

Plant propagation

Seeds were germinated in the University of New Mexico research greenhouse within 3 weeks of collection where they received only natural light. Seeds were sown in flats containing a 4:1:1 mixture of sand, peat and perlite, then individual seedlings were transplanted to 1 L containers within 7 days of germination. Seedlings were kept well watered and received spray irrigation once or twice daily depending on temperatures and evaporative demand. One teaspoon of slow release fertilizer (Osmocote 14:14:14) was applied immediately after transplanting and at four month intervals thereafter. Saplings were grown for one year before measurement of xylem anatomy, by which time all greenhouse-grown saplings had developed woody tissue.

Xylem Vessel Anatomy

We counted a total of 44,671 vessels from72 branches: 8 branches from 5 field populations (high and low latitude *L. tridentata* and *L. divaricata* and high latitude *L. nitida*) and 4 greenhouse-grown populations (high and low latitude *L. tridentata* and *L. divaricata*). Wood was cut into 26 μm sections using a sliding microtome, stained with a 70% solution of Toluidine Blue and wet-mounted on a slide. Sections were magnified 100 x and photographed using a digital camera (Model #E995, Nikon Corporation, Tokyo, Japan) attached to a microscope (Model #Eclipse E400, Nikon Corporation, Tokyo, Japan). We counted all the vessels contained in 4 images of one section from the edge to the center of the section (total image area = 4.4 mm^2), making sure not to include individual vessels in more than one image. This sampling scheme over samples vessels closer to the center of the stem because the same image size covers proportionately more of the area it represents. In addition, there were differences between mean vessel size and number of vessels counted in the innermost images (mean vessel diameter = 14.99 µm, mean number of vessels/mm^2 = 42.5) vs. vessels in the three outer images (mean vessel diameter = 17.52 µm, mean number of vessels/mm^2 = 32.8). However, patterns of vessel size and number were consistent across the sectors when comparing the 9 populations, and removing the innermost images from the analysis did not change the results of the ANOVA model so they were included in the analysis.

For each day that images were generated photographed stage micrometer using the same camera and microscope settings used for stem sections. We used the 'set scale' function of ImageJ (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) to determine the number of micrometers/pixel. Images of xylem were then analyzed in ImageJ using the appropriate scale. They were inverted, then thresholded so that only the vessels were represented by colored pixels. To verify that the particles measured by ImageJ represented actual vessels a drawing of the measured particles, which was generated by the software, was compared to the original .jpg image. When objects appeared in the thresholded image that did not correspond to intact vessels appearing in the original image, such as small cracks in the section or occasional torn vessels, these objects were manually erased and the analysis was repeated. We filtered our particle measurements by the following criteria: shapes touching the edges of the image were excluded, shapes of a circularity less than 0.1 were excluded, and shapes less than 7.5 µm in major diameter of the best fit ellipse were excluded, which corresponds to the lower size limit of vessels measured by Martinez-Vilalta & Pockman (2002). Images were analyzed using the “Analyze Particles” function, which determined an area and the x and y diameters of a best fit ellipse for each individual xylem vessel.

Vessel shape naturally varies from nearly round to ellipsoid, so we calculated vessel diameter from total ellipse area for each individual vessel using the equation

$$\text{vessel diameter} = 2 \sqrt{(\text{Area} / \Pi)} \qquad \text{(Eq. 3-1)}$$

We also report the total number of vessels/mm^2, or the vessel density.

Theoretical hydraulic conductance (K_h) for each vessel was calculated using the equation

$$K_h = \Sigma\ \text{diameter}^4 \qquad \text{(Eq. 3-2)}$$

Next, we calculated vessel size distributions using proc univariate, we placed vessels into 5 μm bins with a left endpoint of 7.5. We report the percent contribution of each size class to the total number of vessels. We also generated distributions for percent contribution of vessels in each diameter class to total theoretical hydraulic conductance. We calculated the sum of hydraulic conductance for all vessels in each diameter class and expressed it as a proportion of total hydraulic conductance for all size classes. Finally, we predicted the level of freeze-thaw embolism by calculating the proportion of total K_h contained in vessels > 42.5 μm in diameter. We chose this diameter as the cut-off for vulnerability of freeze-thaw embolism based on the work of Davis et al (1999) which indicates that vessels 44 μm and larger would be expected to be completely embolized following a single freeze-thaw event when xylem water potential falls to -0.5 MPa or below. We chose 42.5 μm as the lower limit for our study because field water potentials for *Larrea* species are commonly known to fall well below -0.5 MPa, in the range of -2 to -8 MPa, even when soil water potential is near zero (e.g. Franco *et al.* 1994). The critical diameter for freeze-thaw embolism is predicted to decrease as xylem water potential is reduced, therefore we expect that vessels within this range will have a high probability of embolism at water potentials typical of *Larrea* in the field.

Data Analysis

Statistical analysis was performed using SAS version 9.2 (SAS Institute Inc, Cary, North Carolina, USA). For each vessel character (vessel diameter, K_h, vessels/mm^{2,} and predicted freeze-thaw embolism) we generated a mean value for each branch segment and performed statistical analysis on branch means. Predictions of freeze-thaw embolism were arcsine square root transformed to improve normality in the data. We made two ANOVA comparisons; first we compared field and greenhouse grown samples from high and low latitude *L. divaricata* and *L. tridentata* populations (n = 64) for which we considered the effects of species, latitude, growth environment and all their interactions. Because *L. nitida* was only represented by a single, high latitude field population we performed a second ANOVA to test for the effect of species on vessel characters across high latitude field populations of *L. divaricata, L. tridentata and L. nitida* (n = 24). We used proc GLM to generate ANOVA statistics and the lsmeans option to generate p-values for pairwise comparisons using a Tukey correction to control for multiple comparisons.

We also made comparisons of the distributions of vessel diameter and theoretical hydraulic conductance among size classes using proc LOGISTIC. Type 3 analysis of effects were used to determine significance of species, latitude, growth environment and all their interactions as predictors on distributions of field and greenhouse-grown samples from high and low latitude *L. divaricata* and *L. tridentata* populations. A second logistic regression was performed to test for the effects of species on the distributions of field-grown high latitude populations of *L. tridentata*, *L. divaricata* and *L. nitida*. We generated pairwise comparisons of both diameter and K_h distributions using a sequential Bonferonni method (Rice 1989) in which proc FREQ is used determine a P-value for each pairwise combination of the treatment groups, then α values are adjusted to control for the effect of multiple comparisons.

RESULTS

Comparison of L. divaricata and L. tridentata across latitude and growth environment

Vessel diameter

L. tridentata exhibited lower mean vessel diameter in the field than *L. divaricata* (18.20 μm and 18.93 μm, respectively) and in the greenhouse (14.92 μm and 15.17 μm, respectively), but ANOVA revealed no significant effect of species (*Table 3-1*). There were also differences in mean vessel diameter across growth environment; vessel size for greenhouse grown plants was 15.05 μm vs. 18.56 μm for field grown plants and there was a significant effect of growth environment. High latitude populations had smaller mean diameter (16.33 μm) than low latitude populations (17.28 μm) and there was a significant effect of latitude. Among *L. tridentata* high latitude populations had lower mean diameter than low latitude populations when grown in both the field and greenhouse, while *L. divaricata* high latitude plants only had lower mean diameter than low latitude plants when grown in the field, resulting in a significant interaction between latitude and growth environment.

Table 3-1. Results of an ANOVA testing for the effects of species, latitude, growth environment and all their interactions on vessel characters of high and low latitude field and greenhouse populations of L. divaricata and L. tridentata.
** represents significance at α = 0.05; ** at α = 0.01; *** at α = 0.001; **** at α = <0.0001.*

Source of variation	***df***	***Diameter***	***Vessel density***	***Theoretical hydraulic conductance***	***% Kh in vessels > 42.5 μm***
Species	1	1.43	**16.55*****	2.90	1.24
Latitude	1	**5.25***	**12.79*****	**9.63****	**4.01***
Species x Latitude	1	0.45	0.78	2.27	**5.61***
Growth Environment	1	**72.61******	**114.18******	**74.97******	**6.92***
Species x Growth Environment	1	0.34	**8.13****	0.47	3.71
Latitude x Growth Environment	1	**11.25****	2.94	**11.96*****	1.89
Species x Latitude x Growth Environment	1	**5.84***	0.41	**10.99****	3.02

There was also a significant three way interaction between species, latitude and growth environment. Low latitude *L. divaricata* had the smallest mean diameter among greenhouse grown plants (*Table 3-2*) but exhibited the largest differences in mean diameter between greenhouse and field populations, while high latitude *L. divaricata* had the highest mean diameter when grown in the greenhouse (*Table 3-2*) but exhibited the smallest difference across growth environment (*Figure 3-1a*). Among *L. tridentata* plants, mean diameter was higher for low latitude than for high latitude plants in both the greenhouse and field populations (*Table 3-2*), although this difference was more pronounced in field grown plants (*Figure 3-2a*).

Vessel density

Larrea tridentata exhibited significantly higher mean vessel density than *L. divaricata* (mean 144.55 vessels/mm^2 and 113.91 vessels/mm^2, respectively). ANOVA revealed a significant effect of species on vessel density (*Table 1*). There was also a significant effect of latitude, with low latitude plants having higher vessel density than high latitude plants (mean 142.70 vessels/mm^2 and 115.77 vessels/mm^2, respectively). In addition, vessel density was lower for plants grown in the greenhouse (mean 89.00 vessels/mm^2) when compared to those from field populations (mean 169.47 vessels/mm^2). This difference was significant (*Table 3-1*).

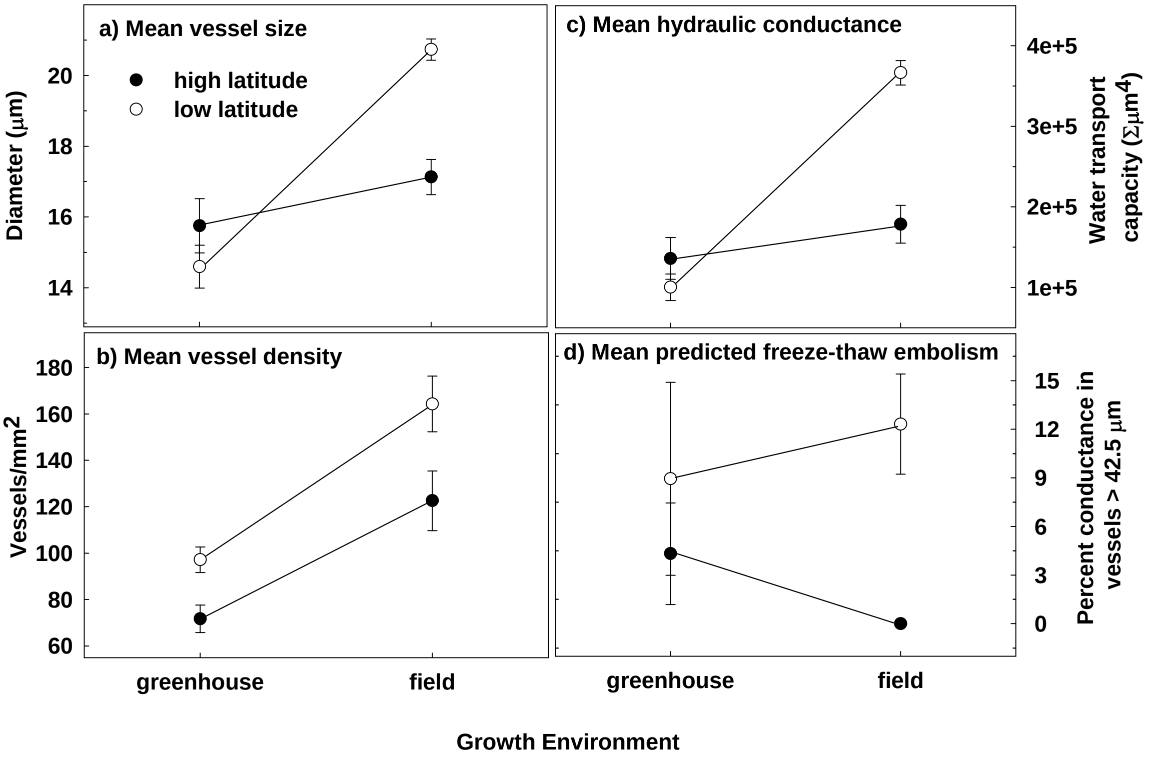
Table 3- 2. Means and standard deviations of vessel characters for the nine combinations of species, latitude and growth environment.
Means with different letters are statistically different in a pairwise comparison using a Tukey correction for multiple comparisons.

Latitude	***Growth Environment***	***Species***	***Diameter***	***Vessel density***	***Theoretical hydraulic conductance***	***% Kh in vessels > 42.5 μm***
High	Field	*L. divaricata*	17.13^{a} +/- 1.41	122.56^{a} +/- 36.34	17.84^{a} +/- 6.59	0^{a}
		L. tridentata	17.67^{b} +/- 1.23	176.53^{bc} +/- 40.06	21.86^{b} +/- 7.80	9.04^{ab} +/- 11.52
	Greenhouse	*L. divaricata*	15.75^{c} +/- 2.17	71.68^{d} +/- 16.83	13.59^{c} +/- 7.34	4.32^{ab} +/- 8.87
		L. tridentata	14.78^{d} +/- 1.19	92.30^{ad} +/- 22.98	8.91^{d} +/- 3.07	0^{a}
Low	Field	*L. divaricata*	20.73^{e} +/- 0.85	164.26^{be} +/- 33.88	36.65^{b} +/- 4.31	12.31^{b} +/- 8.75
		L. tridentata	18.72^{f} +/- 1.77	214.52^{c} +/- 29.63	24.74^{b} +/- 9.49	5.46^{ab} +/- 5.55
	Greenhouse	*L. divaricata*	14.59^{d} +/- 1.71	97.16^{de} +/- 15.59	10.01^{d} +/- 4.67	8.94^{ab} +/-16.84
		L. tridentata	15.06^{d} +/- 2.33	94.86^{ad} +/- 35.40	11.32^{cd} +/- 7.18	0^{a}

The lowest vessel density was observed in greenhouse grown *L. divaricata* from high latitude (*Table 3-2*). High latitude *L. divaricata* also exhibited the lowest vessel density among field grown plants. Though vessel densities were statistically indistinguishable across greenhouse-grown plants, there was a trend toward higher vessel density in greenhouse-grown *L. tridentata* compared to *L. divaricata*. Finally, there were smaller differences in vessel density between greenhouse and field populations of *L.*

divaricata (*Figure 3-1b*) than those observed in *L. tridentata* (*Figure 3-2b*), and there was a significant species by growth environment interaction (*Table 3-1*).

Figure 3-1. Vessel characters in high and low latitude L. divaricata grown in the field and in the greenhouse.
Among greenhouse-grown saplings low latitude plants had higher mean vessel size, mean hydraulic conductance mean vessel density and mean predicted embolism. These same differences were present and strengthed when comparing field populations of low and high latitude plants. Error bars represent one standard error.

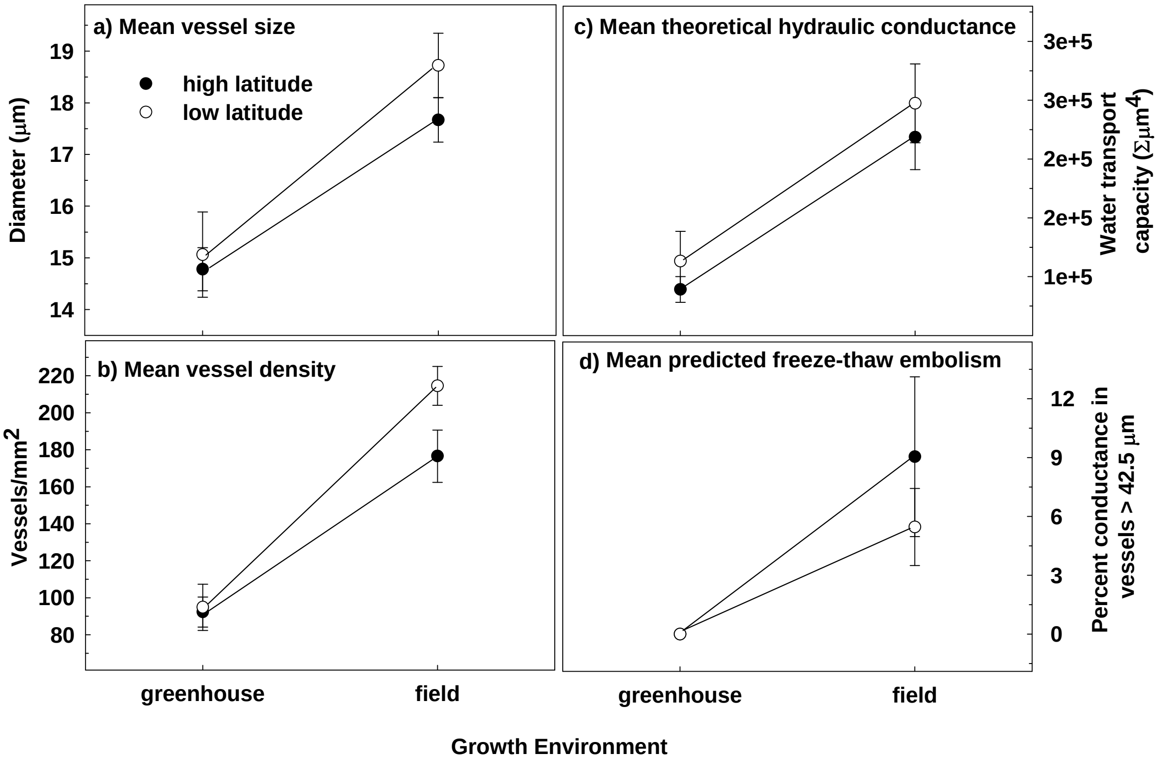


Mean theoretical hydraulic conductance

The differences across latitude and growth environment observed in mean vessel diameter and vessel density were mirrored in comparisons of mean theoretical hydraulic conductance (K_h). Low latitude plants exhibited higher K_h than high latitude plants (mean 20.68 x 10^4 μm^4 and 15.55 x 10^4 μm^4, respectively) and this difference was significant in an ANOVA (*Table 3-1*). In addition, we also observed significantly lower mean K_h in greenhouse-grown plants compared to field-grown plants (mean 10.96 x 10^4 μm^4 and 25.27 x 10^4 μm^4, respectively). ANOVA revealed a significant latitude by growth environment interaction (*Table 3-1*); in *L. divaricata* K_h was higher for high latitude than for low latitude plants when grown in the greenhouse common garden. This difference was significant in a pairwise comparison (*Table 3-2*). This pattern was reversed in field-grown plants, with low latitude plants exhibiting significantly higher K_h than high latitude plants (*Figure 3-1c*). A different pattern was observed in *L. tridentata*, resulting in a significant three way interaction between species, latitude and growth environment (*Table 3-1*). We observed a trend for higher K_h in low latitude *L. tridentata* in both greenhouse-grown and field-grown plants (*Figure 3-2c*), though the differences across latitude were not significant in pairwise comparisons (*Table 3-2*).

Figure 3-2. Vessel characters in high and low latitude L. tridentata grown in the field and in the greenhouse.

Among greenhouse-grown saplings low latitude plants had higher mean vessel size and mean hydraulic conductance than high latitude plants, but there were no differences across latitude in mean vessel density or mean predicted embolism. In field populations these differences were maintained, with the exception of predicted embolism, with high latitude field-grown plants having higher predicted embolism than low latitude field-grown plants. Error bars represent one standard error.



Predicted freeze-thaw embolism

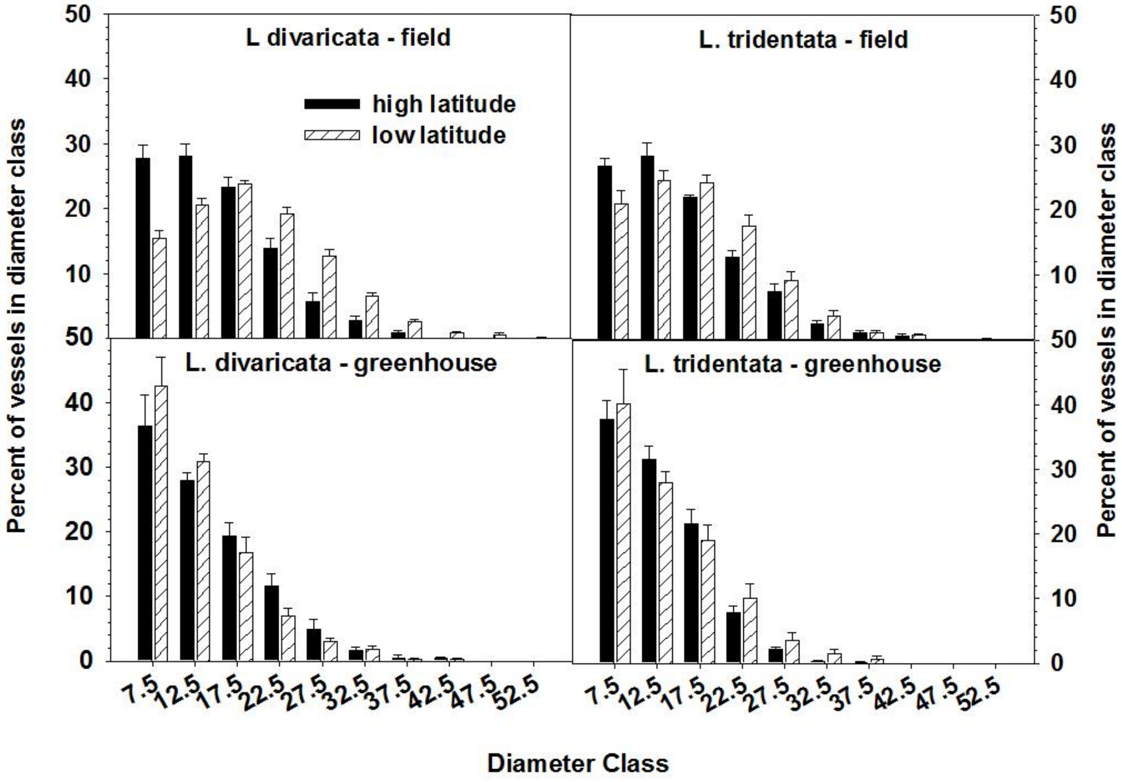
There were large differences across the two growth environments in the percent of K_h maintained in vessels greater than 42.5 μm in diameter, those more likely to embolize following freezing. Greenhouse-grown plants had much lower predicted freeze-thaw embolism than field-grown plants, and these differences were significant in an ANOVA (*Table 3-1*). Mean predicted embolism for greenhouse plants was 3.32% while field-grown plants could experience embolism rates of 6.70% following a single freeze-thaw event. We also detected significant differences across latitude (*Table 3-1*). High latitude plants were predicted to have lower rates of freeze-thaw embolism than low latitude plants (mean 3.34% and 6.68%, respectively). Finally, there were differences between *L. tridentata* and *L. divaricata* across latitude. Low latitude plants of both species were predicted to experience freeze-thaw embolism, while in *L. divaricata* predicted embolism for high latitude plants was zero (*Figure 3-1d*). The differences between high and low latitude *L. divaricata* were significant in a pairwise comparison (*Table 3-2*). In contrast, for *L. tridentata* predicted embolism rate was higher for high latitude plants than for low latitude plants (*Figure 3-2d*), though this difference was not significant in a pairwise comparison (*Table 3-2*).

Distribution of vessel diameters and theoretical hydraulic conductance

We observed differences across species, latitude and growth environment in the number of vessel in each size class (*Figure 3-3*). First, differences between the two species were evident, with *L. divaricata* maintaining more vessels in sizes classes 17.5 μm and above than *L. tridentata*, and logistic regression revealed a significant effect of species (*Table 3-3*). Differences across growth environments were also apparent, with greenhouse-grown plants exhibiting left skewed distributions, maintaining a greater number of vessels in the smallest size classes (below 12.5 μm) than field grown plants. This difference was also significant (*Table 3-3*).

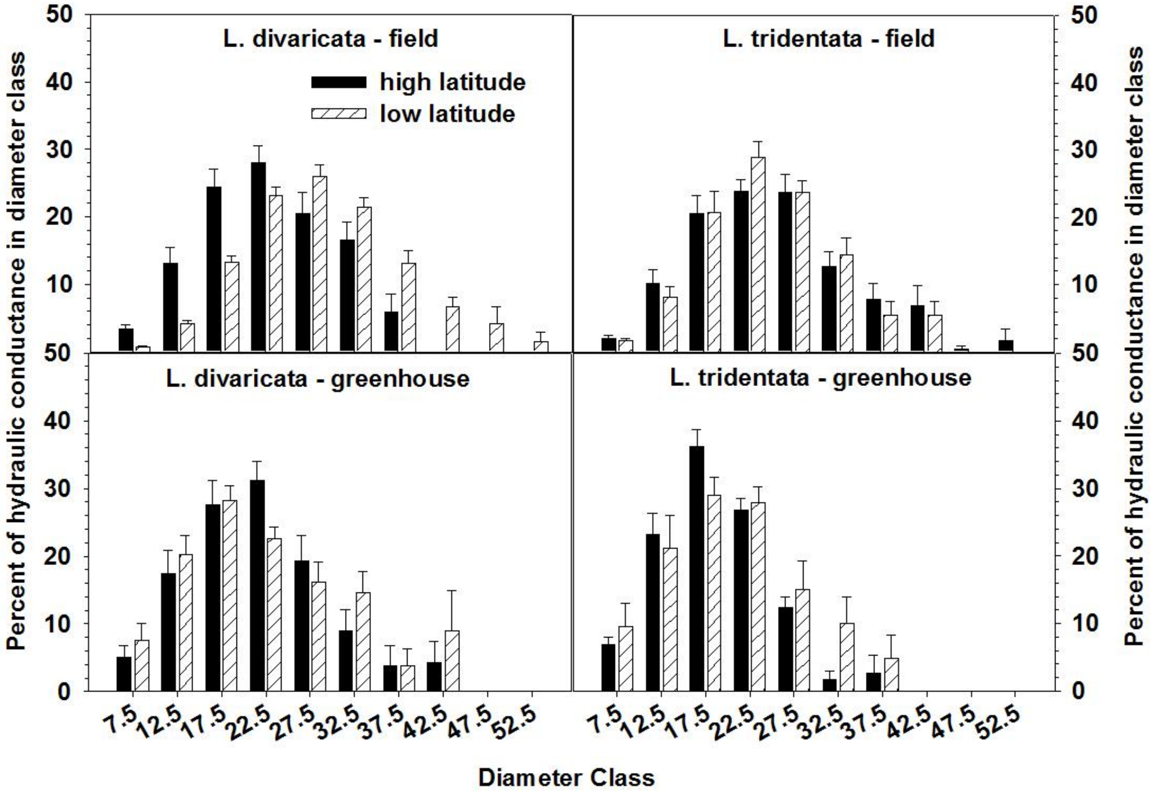
Figure 3-3. Vessel size distributions in field and greenhouse populations of L. divaricata and L. tridentata.

For each size class we calculated the contribution (in %) of vessels in that size class to the total number of vessels all diameter classes. Among greenhouse-grown populations differentiation in vessel size distributions were small, while among field grown populations there were clear distinctions between high and low latitude populations for both species. Among field-grown L. divaricata high latitude plants maintain a larger proportion of vessels in small size classes than low latitude plants, while among field-grown L. tridentata high latitude plants maintained more vessels in the smallest and largest size classes than low latitude plants, with a reduction in the number of intermediate-sized vessels. Error bars represent one standard error.



There was also a significant effect of latitude and low latitude plants exhibiting a greater number of vessels in size classes above 22.5 μm than high latitude plants in combination with a reduced number of vessels in size classes 12.5 μm and below. Sequential Bonferonni comparisons indicated that all measured diameter distributions were significantly different ($P < 0.0001$ for all pairwise comparisons), resulting in significant interactions across nearly all combinations of species, latitude and growth environment (*Table 3-3*). An exception was the lack of a significant combined effect of species and latitude, indicating that latitude influenced the distribution of vessel sizes regardless of species.

Figure 3-4. The distribution of hydraulic conductance in field and greenhouse populations of L. divaricata and L. tridentata.
For each size class we calculated the contribution (in %) of vessels in that size class to the total hydraulic conductance. Among greenhouse-grown populations differentiation in the patterns of investment of water transport were small, while among field grown populations there were clear distinctions between high and low latitude populations among field-grown L. divaricata, with high latitude plants maintain a larger proportion of their water transport capacity in small size classes than low latitude plants, while among field-grown L. tridentata high latitude plants maintained more water transport capacity in the smallest and largest size classes than low latitude plants, with a reduction in the investment in intermediate-sized vessels. Error bars represent one standard error.



Because vessel diameter is used in calculating theoretical hydraulic conductance the differences across distributions of K_h largely reflect those reported for diameter distributions. However, the distribution of K_h also reflect differences in the number of vessels in each size class as well as large differences in water transport capacity of large vs. small vessels, and some interesting patterns emerge when considering these factors in combination. Logistic regression revealed significant differences across species, latitude and environment both independently and across all combinations (*Table 3-3*). Pairwise comparisons indicated that all distributions were significantly different ($P < 0.0001$ for all pairwise comparisons).

Table 3-3. Wald Chi-Square statistics from logistic models testing for differences in vessel size and hydraulic distributions across species, latitude and growth environment. * represents significance at $\alpha = 0.05$; ** at $\alpha = 0.01$; *** at $\alpha = 0.001$; **** at $\alpha = <0.0001$**.**

Source of variation	***df***	***Diameter***	***Theoretical hydraulic conductance***
Species	1	**16.55******	**1.6×10^7******
Latitude	1	**119.00******	**3.4×10^7******
Species x Latitude	1	2.07	**2.7×10^5******
Growth Environment	1	**1858.30******	**3.8×10^8******
Species x Growth Environment	1	**30.73******	**7.2×10^6******
Latitude x Growth Environment	1	**394.77******	**1.8×10^7******
Species x Latitude x Growth Environment	1	**165.04******	**9.6×10^7******

L. divaricata exhibited the predicted pattern across latitude, with both field and greenhouse-grown low latitude plants maintaining greater investment of water transport capacity in size classes greater than 32.5 µm than high latitude plants. Differentiation between low and high latitude *L. divaricata* was strongest in field-grown plants with a shift in investment occurring in the 22.5 – 32.5 µm range (*Figure 3-4*). Among greenhouse-grown plants this pattern was present but less clear, and greenhouse-grown low latitude *L. divaricata* also maintained a larger investment of water transport capacity than high latitude plants in the smallest size classes, those 17.5 µm or less, accompanied by a decrease in the water transport capacity of intermediate size classes (*Figure 3-4*).

Among *L. tridentata* plants only greenhouse-grown plants exhibited the predicted relationship between latitude and the distribution of water transport capacity, with low latitude plants investing more water transport capacity than high latitude plants in vessels greater than 22.5 µm in diameter (*Figure 3-4*). A different pattern was observed among field-grown *L. tridentata*, however. High latitude plants maintained a larger proportion of water transport capacity than low latitude in vessels less than 12.5 µm, but high latitude plants also maintained a greater than expected proportion of water transport in vessels greater than 37.5 µm, accompanied by a decrease in the number of intermediate sized vessels (*Figure 3-4*).

Comparison of high latitude field grown populations

Vessel diameter

Field grown populations of both *L. tridentata* and *L. divaricata* had higher mean vessel diameters (*Table 3-2*) than *L. nitida* (mean diameter 13.98 µm). ANOVA indicated significant differences across species (F = 19.33, *df* 2, $P<0.0001$) and pairwise comparisons determined that mean vessel diameter for *L. nitida* was significantly lower than that for high latitude populations of both *L. tridentata* ($P < 0.0001$) and *L. divaricata* ($P = 0.0002$).

Vessel density

Both *L. tridentata* and *L. divaricata* (*Table 3-2*) exhibited lower vessel densities than *L. nitida* (mean 235.30 vessels/mm^2) and an ANOVA detected a significant effect of species on vessel density (F = 9.57, *df* 2, $P = 0.0011$). Pairwise comparisons indicated that vessel density of *L. nitida* was significantly different from that of *L. divaricata* ($P = 0.0007$) but not from that of *L. tridentata* ($P = 0.0812$).

Theoretical hydraulic conductance

Differences in K_h across the three high latitude populations reflected differences in vessel diameter, with *L. nitida* having the lowest K_h among the three populations (mean K_h 13.56 µm^4) compared to *L. tridentata* and *L. divaricata* (*Table 3-2*). However, large variances in K_h among the three species resulted in no detectable significant differences in an ANOVA comparing this character across species (F = 2.34, *df* = 2, P = 0.1205).

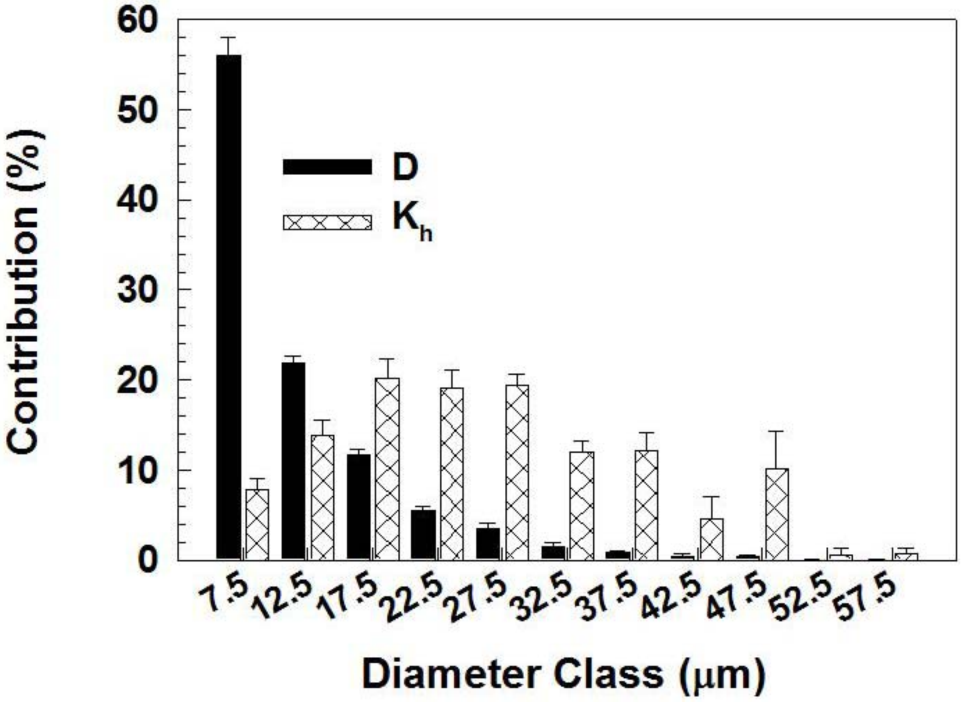
Predicted freeze-thaw embolism

L. nitida had the highest predicted rate of freeze-thaw embolism, 18.40%, for any high latitude field population, and ANOVA revealed a significant effect of species on this character (F = 3.47, *df* = 2, P = 0.0500). Pairwise comparisons indicated that predicted embolism for *L. nitida* was significantly different from that predicted for *L. divaricata* (*Table 3-2*; P = 0.0465) but from that predicted for *L. tridentata* (P = 0.1860).

Distribution of vessel diameters and theoretical hydraulic conductance

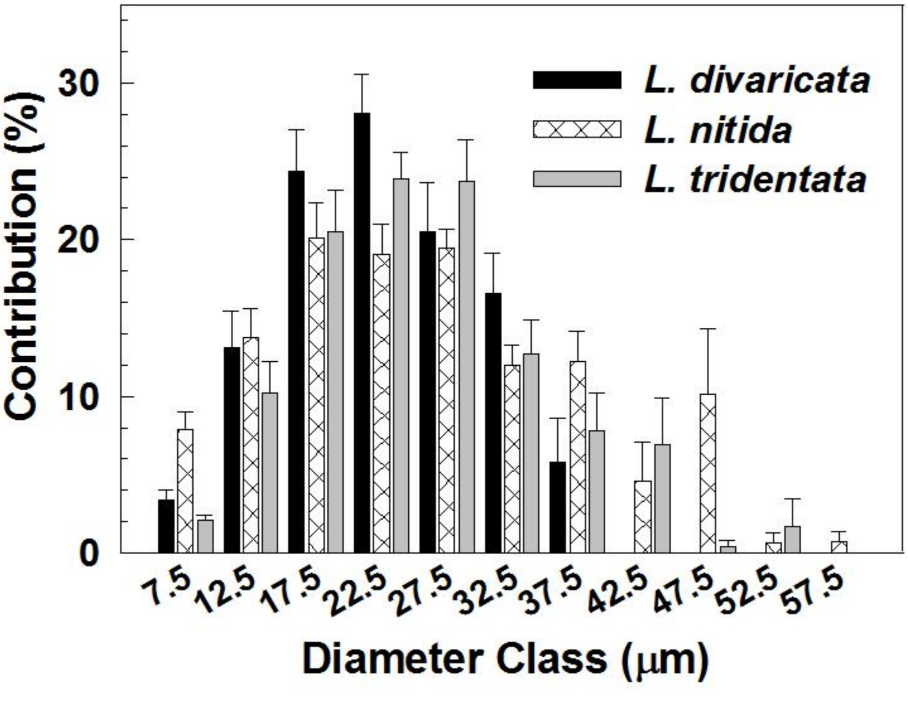
The vessel diameter distribution of *L. nitida* was highly skewed toward smaller size classes, and 79% of the vessels in this population were < 17.5 μm in diameter (*Figure 3-5*). However, *L. nitida* also exhibited the largest vessels measured in the current study, with a few vessels in each size class from 32.5 μm to 57.5 μm. Because of the proportionately greater contribution of these large vessels to total water transport capacity K_h was fairly evenly distributed across all size classes in *L. nitida*, with no more than 20% of K_h in any one size class (*Figure 3-5*).

Figure 3- 5. Vessel size distributions in the high latitude field population of L. nitida. *For each size class we calculated the contribution (in %) of vessels in that size class to the total number of vessels in all diameter classes (D) and to the total hydraulic conductance (Kh). L. nitida maintained a large proportion of vessels in the smallest diameter class, while hydraulic conductance was distributed more evenly across vessel size classes. Error bars represent Standard Error.*



A logistic regression comparing the diameter and K_h distributions of *L. nitida* with those of high latitude field-grown *L. tridentata* and *L. divaricata* revealed significant differences across species for both characters (Wald $X^2 = 1604.16$, *df* = 2, P < 0.0001 and Wald $X^2 = 1.12 \times 10^8$, *df* = 2, P < 0.0001, respectively). In pairwise comparisons both *L. tridentata* and *L. divaricata* were significantly different from *L. nitida* (P < 0.0001 for both comparisons). There were some similarities in the K_h distributions of *L. tridentata* and *L. nitida* that contrast with that of *L. divaricata* (*Figure 3-6*). While *L. divaricata* plants maintained all K_h in size classes 37.5 µm or less, both *L. tridentata* and *L. nitida* invested some of their water transport capacity in vessels 42.5 µm or larger. At the same time, *L. tridentata* and *L. nitida* exhibited equal or greater investment in the smallest size classes, less than 12.5 µm, compared to *L. divaricata*, accompanied by equal or lower investment in intermediate size classes between 17.5 µm and 32.5 µm.

Figure 3-6. Hydraulic conductance in the three high latitude field populations of L. nitida L. divaricata and L. tridentata.
For each size class we calculated the contribution (in %) of vessels in that size class to the total number of vessels in all diameter classes and to the total hydraulic conductance. L. divaricata maintained all water transport capacity in small and intermediate sized vessels < 42.5 µm, while both L. tridentata and L. nitida invested heavily in the smallest and the largest vessel size classes. Error bars represent one standard error.

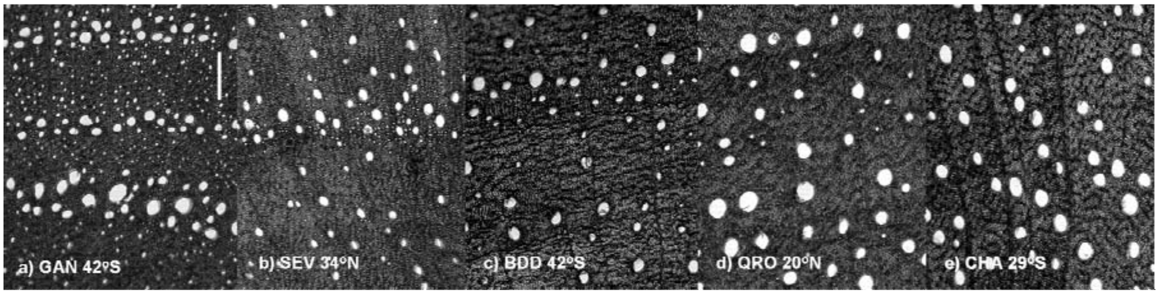


Wood structure

There were qualitative differences in wood structure between the field-grown adults that reflected the quantitative differences just described. Both *L. nitida* (*Figure 3-7a*) and high latitude *L. tridentata* (*Figure 3-7b*) exhibited spatial aggregation of large and small vessels that was hardly detectable in high latitude *L. divaricata* (*Figure 3-7c*) and absent in both low latitude field populations of *L. tridentata* (*Figure 3-7d*) and *L. divaricata* (*Figure 3-7e*). According to the classification scheme presented by Carlquist (2001) high latitude *L. nitida* exhibited ring porous wood structure (Type 4) while high latitude *L. tridentata* exhibited semi-ring porous wood structure (Type 1B). The term semi-ring porous can only be loosely applied to high latitude *L. divaricata*, while low latitude field populations of both *L. tridentata* and *L. divaricata* exhibited decidedly diffuse porous structure.

Figure 3-7. Xylem of Larrea sp. from field collected samples across the five populations.

L. nitida from the high latitude site Gan Gan, Argentina, with a distinctly ring porous growth pattern which was present in all eight samples b) high latitude L. tridentata with a semi-ring porous arrangement, this pattern was present in only 2 of the samples from Sevilleta and was less distinct than that seen in L. nitida, c) L. divaricata from the high latitude site Bajada del Diablo, Argentina, had the smallest vessel sizes of all the field populations and did not exhibit a well defined ring structure. In both low latitude populations, d) L. tridentata from Higurillas, Queretaro, Mexico and e) L. divaricata from Chamical, Argentina we observed distinctly diffuse porous wood, which has previously been reported in L. tridentata by Carlquist (2001). Scale bar = 110 μm



DISCUSSION

Among field grown populations we detected significant differences in all measured wood characters across latitude for both *L. tridentata* and *L. divaricata* in the absence of differences in ploidy, indicating that local adaptation to freezing has occurred in both species. We observed significant differences in mean vessel diameter and mean theoretical hydraulic conductance across greenhouse grown saplings, but only for *L. divaricata*. Although differences between the means of greenhouse grown low and high latitude *L. tridentata* were not significant, the trends we observed were in the same direction as differences seen in the field populations. Significant differences in vessel size and K_h distributions confirm that differences across latitude do exist. Finally, we observed two different types of wood structure in high latitude populations, suggesting that evolutionary responses to freezing have diverged not only across low and high latitude, but among high latitude populations as well.

Our data clearly show that small vessel diameter is common for saplings of this genus. When grown in the common garden environment, mean vessel diameter of saplings only varied between 14.5 μm and 15.9 μm, and not always in the directions predicted by a theory of freezing avoidance. In addition, we saw that freezing tolerance in this genus is likely a combination of genetic and plastic responses. Perhaps more informative are the differences between field grown adults and greenhouse grown saplings within each species by latitude combination. High latitude *L. divaricata* from field populations exhibited characteristics of juvenile wood, but we observed an intriguing parallel between the wood structure of high latitude field populations of *L. tridentata* and *L. nitida*, which showed various degrees of ring-porous structure and an increase in the relative frequency of small and large vessels at the expense of intermediate sizes. These drastically different patterns of allocation to small vs. large vessels and the development of ring-porous structures are likely to have a strong genetic component.

One important factor to consider is that, although mean vessel diameter was lower for field grown high latitude *L. tridentata* than for low latitude plants mean theoretical hydraulic conductance was not statistically distinguishable. This result was due to the higher number of large vessels observed in the high vs. low latitude population and the similar vessel density in the two populations. Reductions in mean vessel diameter are widely reported to be accompanied by an increase in the number of vessels (Carlquist 2001), and for the most part this was true of the populations we measured. However, high latitude *L. divaricata* had the smallest mean vessel diameter and the lowest density. One possible explanation is that this population also faces intense drought pressure, increasing the requirement for structural support which can help prevent vessel implosion (Sperry *et al.* 2008).

Among populations of saplings low rates of freeze-thaw embolism were predicted as little of the theoretical hydraulic conductance was maintained in vessels greater than 42.5 μm, those we expected to be at higher risk of cavitation due to freezing when drought occurs simultaneously (Davis *et al.* 1999). Though mean vessel diameter of saplings was lower than for adults, this was not accompanied by an increase in vessel density, perhaps reflecting the need for small stems to have greater mechanical support. Field grown high latitude *L. tridentata* and *L. nitida* maintained a greater proportion of total hydraulic conductance in these larger vessels, and percent of predicted freeze-thaw embolism of field populations was not statistically different from that predicted for the two low latitude field populations. In fact, the expected loss of hydraulic conductance following freezing was high in high latitude *L. tridentata* and *L. nitida*, though not as great as that predicted for field grown low latitude *L. divaricata*. The high latitude field populations of *L. tridentata* and *L. nitida* maintained vessel densities higher than or similar to low latitude field populations, a reflection of their higher investment in the smallest vessels.

The mean diameter we observed in both low and high latitude field population of *L. tridentata* was lower than the mean diameter of 19.9 μm previously reported for the same high latitude population (Martinez-Vilalta & Pockman 2002). This discrepancy is likely due to differences in sampling methods, as our data contain vessels from across the entire stem, the previous study sampled vessels from only the outmost growth ring. In our study mean diameter for the outermost growth ring was higher (18.4 μm for low latitude and 18.79 μm for high latitude), much closer to their estimate, and they also report a small but significant discrepancy between their measurements of mean diameter in a Sonoran desert population and those reported in Pockman and Sperry (1997), and the magnitude and direction of the difference was similar to the current case. Interestingly, the mean diameter we observed for diploid field grown low latitude *L. tridentata* was 1.8 μm lower than that reported for tetraploid Sonoran desert *L. tridentata* (Pockman & Sperry 1997), where freezing frequency is low. This could be a further indication that polyploidy plays a role in determining vessel size.

Our interpretation of genetic vs. environmental effects is somewhat impaired by the fact that we used samples of different ages for our comparison of greenhouse grown and field grown populations. We saw significant and consistent differences in mean vessel diameter between greenhouse grown saplings and field grown adults, a trend which makes it difficult to distinguish the effects of development from those of the environment. A variety of studies have shown that physiological responses to drought and freezing vary as a function of life-history stage (Sperry & Saliendra 1994; Boorse *et al.* 1998b; Mencuccini & Comstock 1997). Alternatively, the drought resistant woody evergreen *Juniperus osteosperma* exhibited no differences in drought cavitation vulnerability across life-history stages (Linton *et al.* 1998). Ontogenetic variation in wood characters has been previously reported in *Cornus* sp. (Noshiro & Baas 2000) and *Eucalyptus globulus* (Leal *et al.* 2003) and across a variety of angiosperms and conifers from China (Fan *et al*. 2009), while in *Dodonea viscosa* these effects were weak or absent (Liu & Noshiro 2003).

In our case it is clear that ontogenetic effects were present, but we did observe significant differences in vessel diameter, vessel density and hydraulic conductance between saplings, indicating that genetic differentiation in wood characters has occurred between the populations. In addition, the trend for field grown adults from the high latitude *L. tridentata* population to have smaller mean vessel size accompanied by equal or greater numbers of large vessels compared to low latitude *L. tridentata* was mirrored in greenhouse grown saplings. Although a strong environmental component was indicated by the current study, it does provide some evidence for genetic differentiation. Other studies have found that wood characters are genetically and environmentally determined (Fisher *et al.* 2007; February & Manders 1999). A study of 39 species spanning 34 families found that some wood characters were very stable under changing environmental conditions, including inter-vascular pitting pattern, the presence of helical thickenings, pore grouping, perforation plate morphology, fiber types, the occurrence of tracheid and ray histology and morphology (Bissing 1982). In contrast, porosity and vessel grouping pattern were the least stable. This study included *L. tridentata* seedlings which exhibited a shift from the diminishing diffuse porous wood structure (pore diameter gradually decreases toward the outside of the growth ring) observed in field samples to entirely diffuse porous when grown at higher water availability than in their habitat of origin.

If smaller vessel sizes are adaptive in climates where freezing occurs frequently, what could be the advantage to the greater than expected frequency of large vessels we observed in high latitude *L. tridentata* and *L. divaricata*? Ring porous species have been shown to have higher embolism following freezing than co-occurring diffuse porous species (Sperry & Sullivan 1992; Wang *et al.* 1992; Hacke & Sauter 1996). At high latitudes seasonality of the climate may increase, in which case maximizing water use during times of high water availability may effectively maximize growth over the long term (Carlquist 2001) while the production of a large number of small vessels would provide safety as conditions become less favorable (Cochard & Tyree 1990). High latitude *L. tridentata* and *L. nitida* populations included in the current study may improve their ability to compete for water resources by increasing the number of large vessels when water becomes available, but essentially employ a “throw away” strategy when drought and/or freezing are also present. *Larrea* leaves are well known to remain functional low water potential, which could allow them to tolerate drastically reduced hydraulic conductance when large vessels become embolized. High levels of embolism were observed at the high latitude field site currently under discussion by Martinez-Vilalta & Pockman (2002) and Medeiros & Pockman (*unpublished data*), which could not be reconciled with the small mean vessel diameters observed in the species.

Among high latitude *L. divaricata*, the extremely low mean K_h observed in adults may constrain leaf area and limit competitive ability during high water availability. In addition, given the similarities between juvenile and adult wood at this site, the limits of vessel size reduction may have been reached. Simply reducing vessel size may eventually be an evolutionary dead end, as freezing frequency and intensity becomes greater the shift to ring-porous wood structure may allow greater flexibility and further evolution of freeze-tolerant characters. The development of deciduous or freezing deciduous leaf habit could further increase freezing tolerance, and greater sensitivity of leaves to freezing compared to xylem has been reported in the high latitude population of *L. tridentata* (*Chapter 2*).

Although a broad survey of 5329 species found that ring-porous habit is generally associated with deciduousness, occurring in only 1.1% of tropical species (Boura & Franceschi 2007), semi-ring porosity has also been observed in *Zygophyllum dumosum*, another aridland member of the Zygophyllaceae (Baas *et al.* 1983) and in the drought semi-deciduous shrub *Phlomis fruticosa* (Psaras & Sofroniou 2004). In fact, a broader definition of evergreen-ness may be needed to better understand how ring-porous structure could be adaptive in an aridland shrub. Across species of oaks leaves which vary in leaf life span *Quercus ilex* had the longest leaf lifespan, the narrowest vessel diameters and showed the highest freezing tolerance (Cavender-Bares *et al.* 2005). Some "evergreen" species, such as *Cinnamomom camphorum*, exhibit complete leaf turn-over within the course of the year and can only be considered evergreen because they maintain functional leaf area year-round (Boura & Franceschi 2007). Could *L. tridentata* and *L. nitida* that exhibit some degree of vessel size dimorphism at our high latitude field sites have shorter leaf life-spans than those of high latitude *L. divaricata* which produces strictly small vessels? We know of no published data concerning the leaf life span of any *Larrea* species.

Another question for future study concerns the timing of production of large vs. small vessels. Though typical ring porous species produce larger earlywood vessels during the spring, the timing of water availability in the Southern Monte suggests that large vessels may be produced during winter in *L. nitida*, while smaller vessels are produced during the long drought period of summer. A pattern of north-northeast leaf orientation observed in this species indicate that *L. nitida* experiences maximal light interception in the winter, when water availability is highest, and maximal light avoidance (vertical leaf orientation) during summer (Ezcurra *et al.* 1991).

In contrast, high latitude *L. tridentata* exhibits drastically reduced photosynthetic activity during winter (Medeiros, *unpublished data*), and nearly half of the annual precipitation at this site falls during the summer, in the form of intense monsoon rains. This raises the intriguing possibility that larger vessels in this population are produced not in the spring, but in the late summer. In addition, the lack of semi-ring porous structure in some of the high latitude *L. tridentata* samples indicates that larger vessels may not always be formed, and facultative ring-porous growth has been reported in tropical and subtropical plants, especially in the southern hemisphere (Bailey 1944; Wang *et al.* 2005). The stability and plasticity of ring formation as well as the extent to which plants may exhibit deciduous leaf habit in high latitude populations remains an interesting question for contemplation.

CHAPTER FOUR

INTER- AND INTRA-POPULATION VARIATION IN FREEZING TOLERANCE IN HIGH AND LOW LATITUDE *LARREA DIVARICATA* AND HIGH LATITUDE *L. TRIDENTATA*

INTRODUCTION

Population differentiation in freezing resistance has been well documented in wild populations (Pockman & Sperry 1997; Boorse *et al.* 1998b; Feild & Brodribb 2001; Cordero & Nilsen, 2002; Ewers *et al.* 2003; Aranda *et al.* 2005; Stuart *et al.* 2007; Milla *et al.* 2009). Indeed, local physiological adaptation is common (Geber & Griffen 2003; Hereford 2009) and can take place when plants are as little as a few meters apart (Antonovics *et al.* 1971). Furthermore, populations may differ in their capacity for plastic responses to environmental cues (Winn & Evans 1991), and differences across populations in cold acclimation have also been reported (Loik & Nobel 1993; Nardini *et al.* 2000; Loik & Redar 2003; Gianoli *et al.* 2004; Davey *et al.* 2006). However, differentiation across populations growing in the field may be the result of plastic responses in the absence of genetic differentiation, and very little is known about the relative contribution of these two sources of phenotypic variation to the overall variation in freezing tolerance of natural populations.

In recent years a wide variety of evidence has been collected demonstrating a genetic basis for physiological traits (*reviewed in* Geber & Griffen 2003). Physiological changes following stress may be the result of heritable plastic responses, such as the activation of photo-protective mechanisms (Angelopoulos *et al.* 1996; Adams III &

Demmig-Adams 2004), changes in photosynthetic responses (Zangerl & Bazzaz 1983), alteration in allocation patterns (Dudley & Schmidtt 1996; Nicotra *et al.* 1997) and changes in morphology (Wolff & Van Delden 1987). Evidence for inter- and intra-population variation in freezing tolerance can be readily found in the horticultural literature (Aguilera *et al.* 1999; Rapp & Junttila 2001; Jank *et al.* 2002), but there are no data regarding either the heritability or variability of this trait in warm desert shrub populations, and long-lived species have been largely unrepresented in studies of functional trait evolution (Geber & Griffen 2003). These types of data provide insight into the factors that control the evolution of physiological traits in natural populations. Physiological adaptation at ecotones could have large impacts on plant distributions if such adaptations allow for increased productivity and survival. In addition, data regarding the impact of climate variables on keystone species are crucial for generating robust predictions of how ecosystems may respond to changes in climate.

Two species of the genus *Larrea* were chosen for this study because they occur across a wide range of latitudes in North and South America. Members of what one of the most widespread warm desert shrub genera in the Americas, these species define the limits of the warm deserts where they occur. *L. tridentata* is found from 34^{o}N to 20^{o}N (Henrickson & Johnston 1997), and *L. divaricata* found from 46^{o}S to 22^{o}S (Ezcurra *et al.* 1991). Across this range, freezing is common at high latitudes but rare in low latitude populations. Of all the South American species of *Larrea*, *L. divaricata* is the most similar to North American *L. tridentata*, both are diploid and occupy similar habitats (Barbour 1969).

Previous studies of inter- and intra-population genetic variability in *L. tridentata* have found it to be one of the most genetically diverse woody perennials ever measured, (Schuster *et al.* 1994; Cortes & Hunziker 1997; Duran *et al.* 2005). The unpredictable nature of warm desert climates may provide one explanation for this high level of genetic variability (McDonald & Ayala, 1974; Hedrick *et al.* 1976), and plants which face rare, catastrophic events may need to maintain flexible physiological function in order to survive (Schlichting 1989). In fact, a high degree of physiological variation within populations, resulting from high genetic variation, may explain the ability of this species to persist in a wider range of unpredictable environments, as has been postulated for *Sedum wrightii* (Kalisz & Teeri 1986).

Evidence for differentiation in freezing-related xylem characteristics across *L. tridentata* populations has been reported (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002). Though it is clear that population level differentiation in traits related to freezing tolerance has occurred in this species, nothing is currently known about how much of this variation is the result of genetic vs. plastic differentiation across populations or how variation in this trait is partitioned within populations. Variation in physiology, survivorship, size or reproduction within and across populations grown in a common environment provide strong evidence for local adaptation (McGraw & Wulff 1983; Ackerly *et al.* 2000; Arntz *et al.* 2000) and can be useful for distinguishing phenotypic plasticity from genetically based traits (Ackerly *et al.* 2000; Geber & Dawson 2003).

Data concerning the relative contribution of plastic vs. genetic differences in freezing tolerance in natural populations are mixed. Accessions of *Arabidopsis thaliana* collected across its natural latitudinal range and grown in a common garden varied in cold acclimation and freezing tolerance (Zhen & Ungerer 2007), while seedlings of *Artemisia tridentata* from different latitudes grown in a common garden exhibited differences in acclimation but similar freezing tolerance (Loik & Reder 2003). In the tropical tree species *Metrosideros polymorpha* vessel diameter varied across an elevational gradient, but had both genetic and strong plastic components (Fisher *et al.* 2007). Finally, in *Pseudotsuga menziesii*, genetic differentiation in cold tolerance was present along maternal family lines and closely related to minimum temperature (St. Clair 2006), while in *Eucalyptus globulus* genetic differentiation was weak (Tibbits *et al.* 2006).

In order to better understand the proximate causes of differentiation in freezing tolerance observed among high and low latitude populations of warm desert evergreen species, we investigated responses to freezing of 3 month old seedlings in 10 maternal families from each of three populations, one low and one high latitude population of *L. divaricata* and one high latitude population of *L. tridentata*. We measured chlorophyll fluorescence, survival and projected plant area before freezing, immediately after or 24 hours after freezing and one week following freezing in high and low latitude populations of *L. divaricata* and a high latitude population of *L. tridentata*. For *L. divaricata* we also made measurements of F_vF_m before and after acclimation. We calculated the heritability of F_vF_m and projected plant area before and after freezing.

We asked the following questions: 1) Is there genetic differentiation between populations from habitats with different mean annual temperature? 2) Is freezing tolerance of seedlings correlated with long-term minimum temperature at their site of origin? 3) What is the potential for further freezing adaptation in both high and low latitude populations? We expected that inter-population variation in freezing tolerance would be highest when comparing high latitude to low latitude plants. Specifically, high latitude seedlings should be more freezing tolerant than low latitude seedlings and have greater acclimation potential. Alternatively, seedlings from different latitudes grown in a common garden environment may have equal freezing tolerance, suggesting that differentiation in tolerance between field populations may be the result of plastic responses to growth environment, rather than genetic differentiation. If plastic responses are important for freezing adaptation intra-population variation in freezing tolerance may be low. In addition, intra-population variation in freezing tolerance may be lower at high latitudes than at low latitudes because selection for freezing tolerance has already taken place where freezing is more intense and/or frequent.

MATERIALS AND METHODS

Field sites

High latitude L. tridentata LTER, New Mexico, USA

The high latitude North American site was located at the edge of the distribution of *L .tridentata*, near the Five Points area of the Sevilleta Long Term Ecological Research site (34º 20'N 106º 45'W) at an elevation of 1610 m. Mean annual precipitation is 215 mm ranging from 86 to 306 mm, with more than typically occurring 50% during the North American monsoon (July – September). Mean annual temperature is 9°C and long-term minimum temperature ranges between -14ºC and -20ºC (Moore 1989-2009).

Low latitude L. divaricata INTA Station, La Rioja, Argentina

The low latitude South American site is located in at the Northern end of the Monte Desert (29º 25’ S 66º52’ W) at an elevation of 517 m. The mean annual temperature (1931 -2005) is 20ºC (www.globalbioclimatics.org). The northern edge of the distribution of *L. divaricata* occurs in the province of La Rioja where mean annual precipitation is 100-200 mm depending on slope and aspect. Mountain valleys experience only a handful of nights each month below zero in June, July, and early August with long-term minimum temperature of -7 or -8°C. (P. Searles, *personal communication*).

High latitude L. divaricata, Chubut, Argentina

The high latitude South American site is located at the far southern end of the Monte Desert (42°31’ S 68°15’ W). Climate data are sparse in this region, however, the climate is cold arid; mean annual precipitation is around 200 mm, with most of this moisture arriving in the winter (Ezcurra *et al.* 1991).

Seed collection and plant propagation

Seeds from 30 maternal plants were collected at each site. Seeds from the high latitude *L. tridentata* population were collected on October 21st, 2007, while seeds from low and high latitude *L. divaricata* populations were collected on June 8th and 20th, 2008, respectively. Seeds were germinated in flats containing 4:1:1 sand, peat moss and pearlite within 3 weeks of collection and watered twice daily until transplanting. Seeds were not stored and grown at the same time because stored seeds of this genus are notoriously recalcitrant and it is very difficult to obtain the large number of seedlings needed for the experiment from stored seeds (McGee & Marshall 1993). There were large differences between maternal families in seedling emergence with approximately 20-25 families from each population having moderate to high rates of emergence (upwards of 30% of seeds germinating) and between 5-10 families from each population having low rates of emergence (less than 1% of seeds germinating); only families with moderate to high emergence were considered for study families. Seedlings were transplanted into 20 cm^3 cone-tainers containing the same soil mixture before the first set of true leaves emerged. Following one week of growth in the 20 cm^3 pots the 10 families with the highest survival were selected as study families. Plants were then grown in the University of New Mexico greenhouse for 3 months. For high latitude *L. tridentata* seedlings, only two blocks were used and plants were randomized across maternal families four weeks before temperature treatments began. For high and low latitude *L. divaricata* plants, 4 blocks were used and plants were randomized across maternal families and site immediately upon transplanting (approximately 1 week following germination). Blocks were randomized once per week and the entire experiment took up a 3 m x 3 m space. Seedlings were watered with 1/4 strength Peters 20:20:20 (The Scotts Company, Marysville, OH, USA) every two weeks.

Low Temperature Treatments

Cold acclimation took place in a ConViron growth chamber (Model #E8, Controlled Environments Limited, Winnipeg, Canada) for 7 days prior to freezing treatments. Plants experienced nighttime temperatures of 1^{o}C and daytime temperatures of 12^{o}C. Humidity was controlled below 50%. Light was provided 12 hours per day using sodium halide lights at an intensity of 1160 µmol m^{-2}. Because root freezing may have a significant impact on plant function, freezing took place in a chamber designed to freeze plants intact in their pots without exposing roots to low temperatures (*methods described in Chapter 2*). This procedure allowed exposure of only the canopy portion of the plant to low temperatures, while maintaining a temperature of 6^{o}C in the root zone. High latitude *L. tridentata* seedlings experienced freezing to either -10^{o}C or -12^{o}C. These temperatures were chosen because long-term temperature records from the high latitude *L. tridentata* site at the Sevilleta LTER indicate that minimum temperatures between -9^{o}C and -12^{o}C are typically reached 2-3 times per month during the months of January and February. Using data collected by Doug Moore 1999-2005 at the Five Points met station at the Sevilleta Long Term Ecological Research site, we determined the number of days the temperature reached a minimum between -9^{o}C and -12^{o}C at 7AM. Preliminary tests indicated that -12^{o}C resulted in nearly 100% mortality in plants from South American populations, and so temperature treatments were shifted to -10^{o}C and -8^{o}C. Low temperature treatments were immediately followed by measurements of physiological and morphological parameters, then plants were returned to the greenhouse.

Chlorophyll fluorescence

In order to quantify stress experienced by seedlings as a result of freezing treatments we used measurements of dark adapted chlorophyll fluorescence (F_vF_m). F_vF_m is a non-invasive measurement allowing for repeated measurements of the maximum quantum yield of PSII in the dark over time on the same leaves, and it has been shown to correlate strongly with leaf dieback (Boorse *et al.* 1998a) and with a more traditional, but destructive measure of freezing damage, electrolyte leakage (Ehlert & Hincha 2008). We used a PSI Open Flurocam (Model# FC 1000-H, Photon Systems Instruments, Brno, Czech Republic). The seedlings of *Larrea* are somewhat two dimensional, presenting very little profile from a 90^o angle, so plants were gently sandwiched between two pieces of Plexiglas for 1-2 minutes during imaging to present one of the flat sides of the plant to the camera. In previous experiments this technique showed no negative effects on plant survival and helped to ensure that images were made of the same leaves and at a uniform height at each measurement period, thus making our comparisons of F_vF_m over time more robust. Once in the plexiglass sandwich, one side of the entire plant was imaged. The leaf area imaged by the camera represented approximately one-quarter of the total leaf area of the plant.

Plants were kept in the dark for at least 8 hours before measurements were made, and measurements were completed between the hours of 4AM and 8AM. Care was taken to maintain the seedlings in darkness while transferring them to the measurement apparatus. We calculated F_v from the equation:

$$(F_m - F_o)/\ F_m \qquad \text{(Eq. 4-1)}$$

where F_o is the initial fluorescence and F_m is the maximum fluorescence (Maxwell & Johnson 2000). We report the numeric average of F_vF_m (F_v / F_m) for the entire plant, which includes leaves and stems. Measurements were made before freezing (all populations), immediately after 7 days acclimation (*L. divaricata* only), immediately after freezing (*L. tridentata* only), 24 hours after freezing (*L. divaricata* only) and one week after freezing (all populations).

Flash Train Analysis, which is performed to determine if the saturating pulses provided by the fluorescence system in use is sufficient to induce maximum fluorescence in *L. tridentata* seedlings without causing damage, was measured in the light using the flash-train method described in (Loriaux *et al.* 2006). A saturating light pulse was applied at 5 different intensities and the relationship between 1/light intensity (1/Q) and measured F_m' was determined. The y-intercept then predicts maximum F_m'. For each individual plant a predicted F_m' is calculated, and the percent deviation of the measured F_m' from predicted F_m' is calculated. Light adapted chlorophyll fluorescence data was collected on 45 juvenile *L. tridentata*, we found that all measured values of F_m' were within +/- 5% of predicted F_m', indicating that our saturating pulse provided sufficient light to saturate photosystem II without causing damage.

Projected plant area was calculated from fluorescence images generated by the PSI software. Leaf area was calculated using Scion Image (Scion Corporation, Frederick, Maryland, USA) from an equation relating pixel area to leaf area.

Survival

Survival was monitored for one month following freezing. Because plants that died following freezing did so within a few days we only report two week survival data.

Data analysis

Data analyses were performed using SAS (Ver. 9.1, SAS Institute Inc., Cary, North Carolina, USA). We tested for a variety of differences between populations in F_vF_m and projected plant area using PROC GLM to generate ANOVAs. For all analyses non-significant interactions with block were removed from models to conserve degrees of freedom and maternal family was specified as random variable. To compare F_vF_m of high and low latitude *L. divaricata* plants following cold acclimation we used the model: fvfm = population, block and their interaction. To test for differences within and across populations in responses to freezing we used time series analyses, which included only plants which survived freezing, we used the model: F_vF_m pre-freeze and F_vF_m post-freeze, projected plant area pre-freeze and projected plant area post-freeze = population and block. For comparisons across maternal families we used the model: F_vF_m pre-freeze and F_vF_m post-freeze, projected plant area pre-freeze and projected plant area post-freeze = family, minimum temperature, block and the interaction between family and minimum temperature. We also computed Pearson correlation coefficients for all measured characters. To test for differences in survival across populations following freezing to -10^{o}C we used PROC LOGISTIC using the model survival = block, population, minimum temperature and the interaction between population and minimum temperature. To test for differences across maternal families we used the model survival = family, minimum temperature and block.

Broad sense Heritability Calculations

Broad sense heritability and the standard errors reported are intra-class correlation coefficients which were calculated separately for each population using the method of Geber & Dawson 1997) from mean squares generated by SAS proc GLM for the model: family = F_vF_m and projected plant area using the equation

$$MS_{family}/MS_{error} + MS_{family} \qquad \text{(Eq. 4-2)}$$

Broad sense heritability measured on seedlings grown in a controlled environment is known to provide inflated estimates of heritability (Geber & Griffen 2003), so estimates presented here should be considered generous upper bounds.

RESULTS

Survival

High latitude *L. tridentata* seedlings had the highest proportion of plants surviving following freezing to -10^{o}C, followed by high latitude *L. divaricata*. Within the low latitude *L. divaricata* population approximately one quarter of seedlings died following freezing to -10^{o}C. (*Figure 4-1)* and there were significant differences across populations

(*Table 4-1*). Only the low latitude *L. divaricata* population exhibited differences in freezing survival across maternal family lines, with some families experiencing 100% mortality and others with greater than 40% survival (*Figure 4-2*). These differences were significant (*Table 4-1*), as was the effect of minimum freezing temperature, and there were large differences in survival between the -8^{o}C and -10^{o}C treatment. There was also a significant effect of minimum temperature on survival in the high latitude *L. divaricata* population.

Table 4-1. Wald Chi-Square statistics from logistic models testing for differences in survival across populations and maternal families of L. divaricata and L. tridentata. *(a) across all three populations following freezing to -10^{o}C and (b) across maternal families in the three populations following freezing to -10^{o}C or -8^{o}C in L. divaricata and to-12^{o}C or -10^{o}C in L. tridentata. * represents significance at P = 0.05, ** at 0.01 and *** at <0.0001.*

	Source of variation	*df*	X^2
Across Populations			
	Population	2	**26.19*****
	Minimum temperature	1	**52.22*****
	Block	1	2.09
Across Maternal Families			
Low latitude *L. divaricata*	*Family*	9	**18.95***
	Minimum temperature	1	**20.88*****
	Block	1	3.62
High latitude *L. divaricata*	*Family*	9	3.71
	Minimum temperature	1	**16.83*****
	Block	1	1.00
High latitude *L. tridentata*	*Family*	9	0.02
	Minimum temperature	1	0.10
	Block	1	0.02

Figure 4-1. Survival across populations of high and low latitude L. divaricata and high latitude L. tridentata.

Both high and low latitude L. divaricata exhibited lower survival following freezing than L. tridentata, although low latitude L. divaricata had the lowest survival rate following freezing to -10^{o}C.

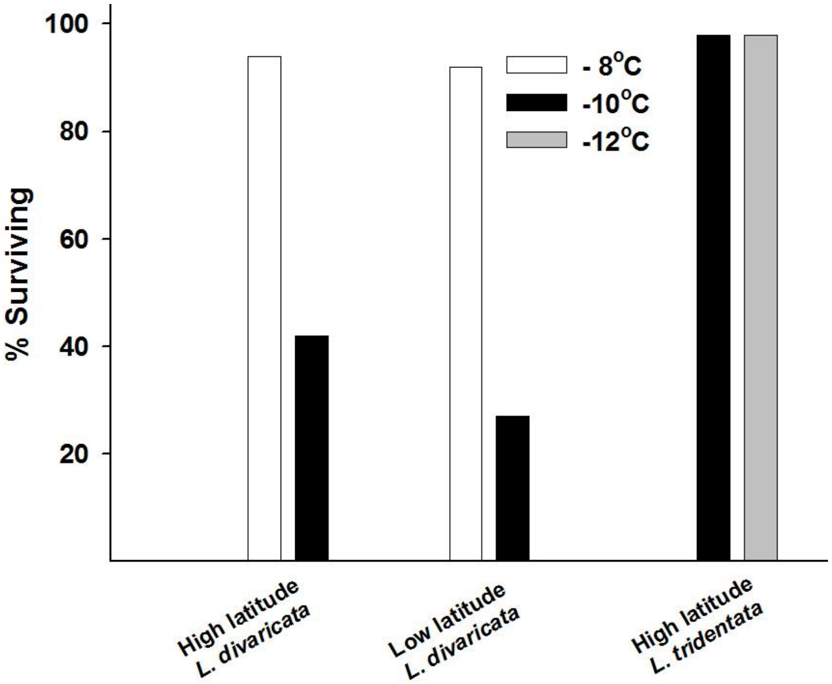
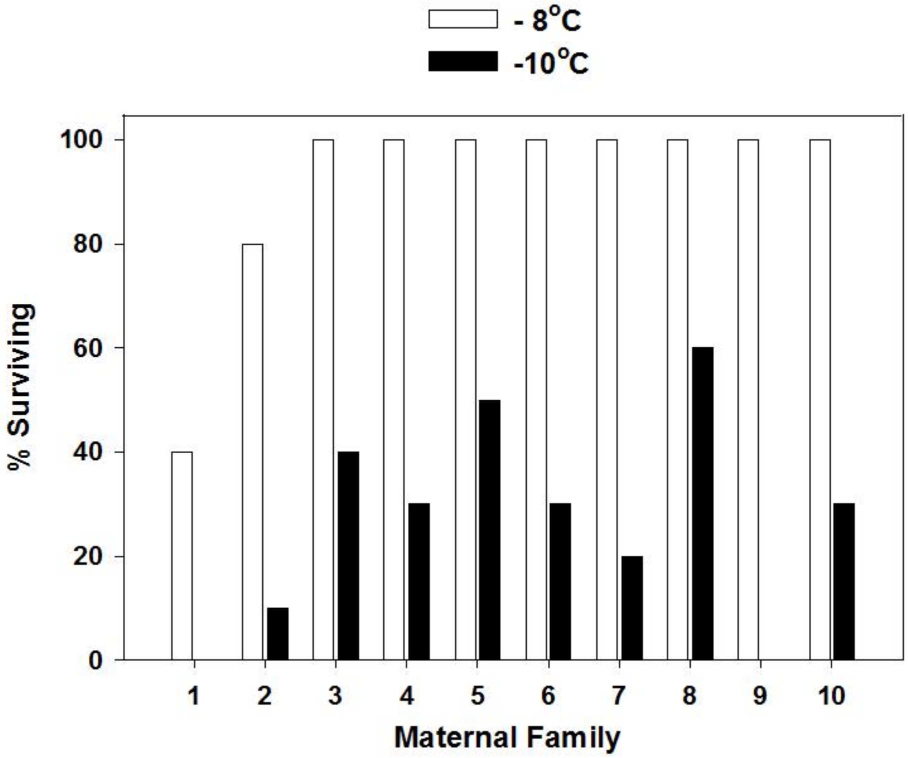


Figure 4-2. Survival across low latitude L. divaricata maternal families.
Survival was high following freezing to -8°C, with all maternal families exhibiting 40% or greater survival. However, following freezing to -10°C no maternal family had higher than 60% survival and some families experienced 100% mortality.



Responses to cold acclimation

Mean post-acclimation F_vF_m was higher for low latitude *L. divaricata* (0.40) than for high latitude *L. divaricata* (0.36), and there was a significant effect of acclimation on F_vF_m (Time effect; *Table 4-2*). However, there was not a significant time x population interaction, as both populations showed post acclimation reductions in F_vF_m in proportion to pre acclimation F_vF_m. There was an effect of block on F_vF_m following acclimation, but this was driven by seedlings in the high latitude *L. divaricata* population, which exhibited a significant effect of block when examined separately in the within population analysis (*see below*).

In the high latitude *L. divaricata* population, there was some variation in post acclimation F_vF_m which ranged from 0.30 to 0.40, but these differences were not partitioned across maternal family lines. A significant time x block interaction in the high latitude *L. divaricata* population (*Table 4-2*) indicates that environmental variation in the greenhouse did play a role in the response of this population to cold. There was no block effect and less variation in F_vF_m in the low latitude *L. divaricata* population with mean post-acclimation F_vF_m ranging from 0.37 to 0.44 across families, and there were significant differences across maternal families in the response to cold acclimation (*Table 4-2*; Time x family interaction).

Table 4-2. Results of time series ANOVAs testing for differences in responses to freezing across populations and across maternal families of L. divaricata and L. tridentata.
*(a) across all three populations following freezing to -10^{o}C and (b) across maternal families in the three populations following freezing to -10^{o}C or -8^{o}C in the L. divaricata populations and to-12^{o}C or -10^{o}C in the L. tridentata population. * represents significance at P = 0.05, ** at 0.01 and *** at <0.0001.*

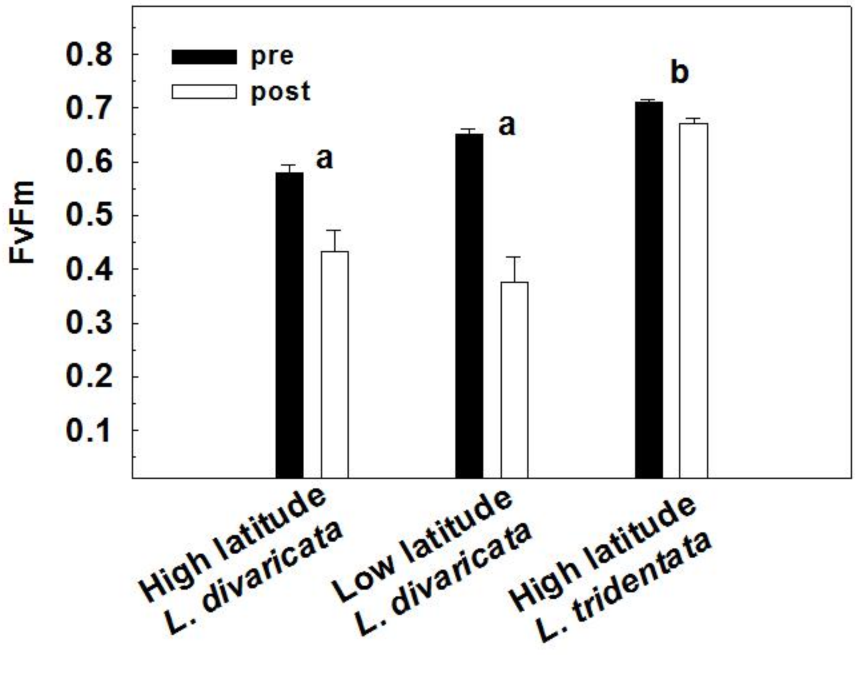
		Source of variation	*df*	**F_vF_m**	**Projected Plant Area**
		Across Populations			
Following cold acclimation		*Time*	1	**1417.27*****	-
		Time x Population	1	0.13	-
		Time x Block	3	**3.49***	-
Following freezing to -10^{o}C		*Time*	1	**78.88*****	**57.55*****
		Time x Population	1	**8.85****	1.73
		Time x Block	4	0.53	0.45
		Across Maternal Families			
Following cold acclimation	**Low latitude *L. divaricata***	*Time*	1	**1026.30*****	-
		Time x Family	9	**2.12***	-
		Time x Block	3	1.33	-
	High latitude *L. divaricata*	*Time*	1	**639.45*****	-
		Time x Family	9	1.68	-
		Time x Block	3	**6.03****	-
Following freezing	**Low latitude *L. divaricata***	*Time*	1	**47.73*****	**74.67*****
		Time x Family	9	1.18	**2.11***
		Time x T_{min}	1	**7.86****	**6.73***
		Time x Family x T_{min}	7	0.31	**2.53***
		Time x Block	3	0.07	0.04
	High latitude *L. divaricata*	*Time*	1	**21.31*****	**63.43*****
		Time x Family	9	0.64	0.68
		Time x T_{min}	1	1.30	1.25
		Time x Family x T_{min}	9	1.32	0.99
		Time x Block	3	0.22	0.28
	High latitude *L. tridentata*	*Time*	1	**14.90****	**56.09*****
		Time x Family	9	0.44	**3.33****
		Time x T_{min}	1	0.08	**8.55****
		Time x Family x T_{min}	9	1.07	**2.23***
		Time x Block	1	0.31	1.34

Responses to freezing

Seedlings from the high latitude *L. tridentata* population had the highest F_vF_m and projected plant area before freezing and exhibited only a slight drop in F_vF_m one week following freezing, while low latitude *L. divaricata* seedlings exhibited the greatest difference between pre and post-freeze characters (*Figure 4-3*). However, the differences between the populations in the freezing response of projected plant area (*Figure 4-4*) were not as great as they were for F_vF_m, resulting in no significant differences (*Table 4-2*). Among high latitude *L. tridentata* and low latitude *L. divaricata* populations there were significant differences in projected plant area following freezing across maternal families, minimum temperature and their interaction (*Table 4-2*).

Figure 4-3. Dark adapted chlorophyll fluorescence before and after freezing in high and low latitude L. divaricata and high latitude L. tridentata.

Both high and low latitude L. divaricata exhibited lower F_vF_m following freezing than L. tridentata, although low latitude L. divaricata had the lowest F_vF_m following freezing to -10°C.Letters represent significant differences in a pairwise comparison, error bars represent one standard error.



Within the high latitude *L. tridentata* population two size phenotypes were observed, with some maternal families having approximately twice the mean projected plant area of other maternal families, yet families with larger mean projected plant area showed the greatest reductions in plant area in response to freezing (*Figure 4-5*). This pattern was present but less defined in the low latitude *L. divaricata* population (*data not shown*) partly because of high variability within families following freezing to -10°C. In the low latitude *L. divaricata* population there were also differences in the response of F_vF_m to -8°C vs. -10°C, and reductions in F_vF_m of survivors from this population were greater following freezing to the colder temperature. There were no differences across families in the response of F_vF_m to freezing for any population, and no differences in response between families of the high latitude *L. divaricata* population for any character.

Figure 4-4. Projected plant area before and after freezing in high and low latitude L. divaricata and high latitude L. tridentata.

Both high and low latitude L. divaricata experienced greater reductions in plant area following freezing than L. tridentata, although low latitude L. divaricata exhibited the greatest reductions in plant area following freezing to -10°C. Letters represent significant differences in a pairwise comparison, error bars represent one standard error.

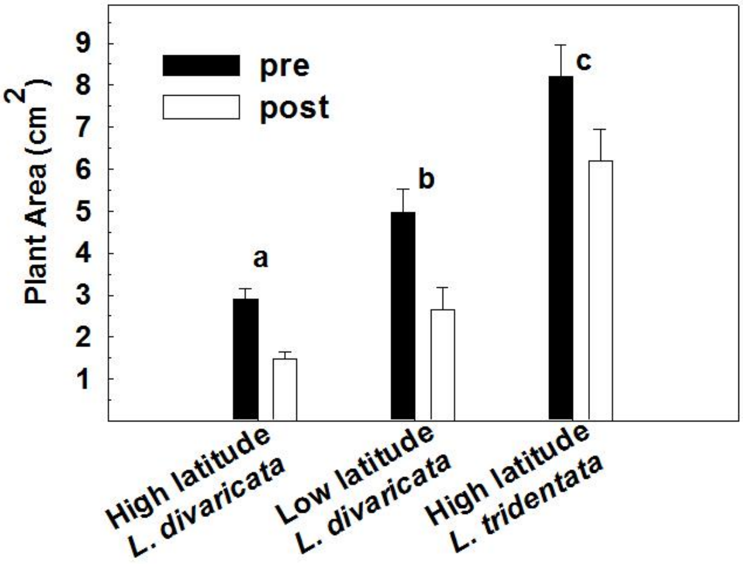
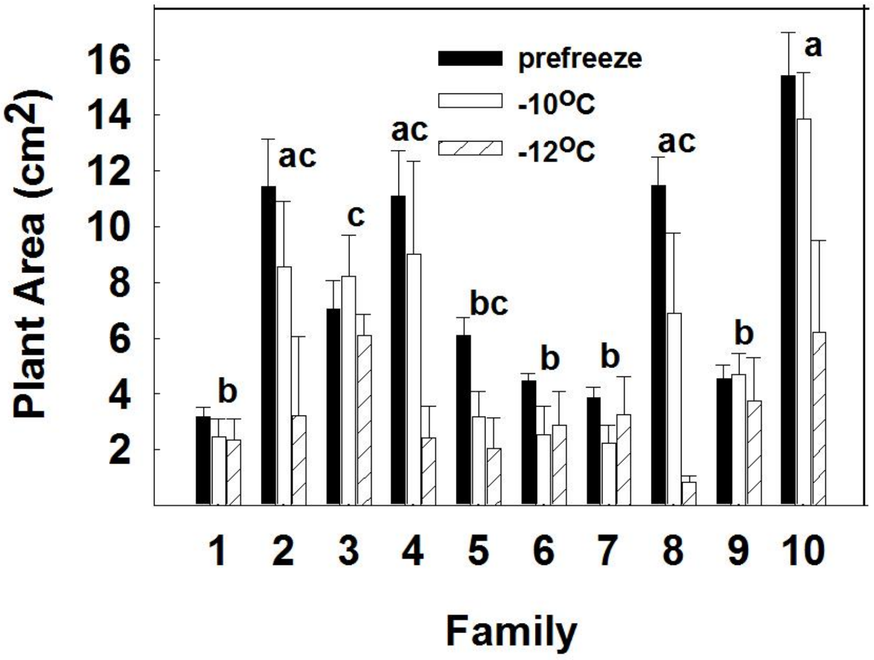


Figure 4-5. Comparisons of projected plant area across maternal families from high latitude L. tridentata.
Before freezing two size phenotypes were observed, with some maternal families producing large seedlings and some maternal families producing small seedlings. Following freezing to -12°C maternal families with small seedlings showed very little reduction in mean plant area, while those with large seedlings exhibited larger reductions in mean plant area. Letters indicate significant differences between groups, error bars represent standard error.



Correlations between characters measured before and after freezing

There were significant positive relationships between pre-treatment F_vF_m and all subsequent measurements, indicating that plants which had higher F_vF_m before temperature treatments did better following cold acclimation and freezing (*Table 4-3*). This was not true for pre-freeze projected plant area, which was positively correlated with pre F_vF_m, one week post F_vF_m and projected plant area, but weakly negatively correlated with performance after acclimation and shortly after freezing, indicating that bigger plants had an advantage in recovery from freezing but not in the time period immediately following acclimation or freezing. There were no significant correlations between post acclimation F_vF_m and any character, suggesting that that these two species exhibit a very weak acclimation response

Table 4-3. Pearson Correlation Coefficients for physiological and morphological characters of seedlings measured before and after freezing.
*Includes both surviving and non-surviving plants, where non-survivors were assigned a value of zero. * represents a significant correlation at P = 0.05, ** at 0.01 and *** at <0.0001.*

	Prefreeze F_vF_m	**Post acclimation F_vF_m**	**Immediately postfreeze F_vF_m**	**24 hours postfreeze F_vF_m**	**One week postfreeze F_vF_m**	**Pre freeze Projected plant area**
Pre freeze F_vF_m	1	-	-	-	-	-
Post acclimation F_vF_m	0.58***	1	-	-	-	-
Immediately postfreeze F_vF_m	0.34**	NA	1	-	-	-
24 hours postfreeze F_vF_m	0.16**	0.12	NA	1	-	-
One week postfreeze F_vF_m	0.47***	0.19	0.63***	0.87***	1	-
Pre freeze Projected plant area	0.33***	0.01	-0.1	-0.06	0.23**	1
One week post Projected plant area	0.33***	0.08	0.45***	0.44***	0.38***	0.61***

Broad Sense Heritability

We observed a broad range of H^2 across populations and measurement times, from 0.28 to 0.94 (*Table 4-4*). Before freezing H^2 of both F_vF_m and projected plant area was above 0.43 for all populations and was greatest for projected plant area, which exhibited the largest H^2 observed during the study. Pre-freeze H^2 was highest for the high latitude *L. tridentata* population and lowest for high latitude *L. divaricata* seedlings, and this pattern was maintained post-freeze. For both high latitude *L. tridentata* and low latitude *L. divaricata* seedlings H^2 was lowest following the coldest freezing treatments (-12^{o}C for high latitude *L. tridentata* and -10^{o}C for low latitude *L. divaricata*). This pattern was reversed for high latitude *L. divaricata* seedlings, which exhibited the lowest observed H^2 following freezing to -8^{o}C. For both low and high latitude *L. divaricata* populations H^2 following cold acclimation was intermediate and identical.

Table 4-4. Broad sense heritability estimates for seedling physiological and morphological characters.

Time	**Character**	***High latitude L. tridentata***		***High latitude L. divaricata***		***Low latitude L. divaricata***	
Pre treatment	F_vF_m	0.70 +/- 0.05		0.43 +/- 0.03		0.67 +/- 0.03	
	Projected Plant Area	0.94 +/- 0.05		0.69 +/- 0.04		0.79 +/- 0.03	
Post Cold Acclimation	F_vF_m	-		0.45 +/- 0.04		0.45 +/- 0.04	
Post Freeze	*Minimum Temperature*	- 12^{o}C	-10^{o}C	-10^{o}C	-8^{o}C	-10^{o}C	- 8^{o}C
	F_vF_m	0.49 +/- 0.08	0.62 +/- 0.09	0.40 +/- 0.09	0.34 +/- 0.10	0.41 +/- 0.14	0.53 +/- 0.09
	Projected Plant Area	0.45 +/- 0.08	0.81 +/- 0.10	0.49 +/- 0.10	0.28 +/- 0.09	0.46 +/- 0.14	0.60 +/- 0.10

DISCUSSION

We observed differentiation within and among the two species of *Larrea*, with *L. tridentata* having higher freezing tolerance than *L. divaricata*, and a trend for high latitude *L. divaricata* to have higher freezing tolerance than low latitude *L. divaricata*. Adult *L. tridentata* growing at the Nevada Desert Face Facility exhibited reduced F_vF_m below 0.5 following freezing to -10^{o}C, while soil was wet (Naumburg *et al.* 2004). In addition, young seedlings from a California population of *L. tridentata* experienced mortality following a temperature treatment equivalent to the acclimation treatment we applied (Yang 1967), further indicating that genetic divergence in freezing tolerance has occurred in the genus *Larrea*. We also observed differences between maternal families following freezing in the low latitude population of *L. divaricata* and the high latitude population of *L. tridentata*. This was not the case in the high latitude population of *L. divaricata*, further suggesting that local adaptation to freezing may have already occurred in this population, as strong selection can reduce overall genetic variation (Ackerly *et al.* 2000).

There was also a significant interaction between plant size and freezing tolerance, and we saw that size was positively correlated with post-freeze F_vF_m and survival one week after freezing, but this relationship was reversed in the first 24 hours following freezing, indicating that larger plants experienced greater stress from freezing but were able to recover more quickly. Though high latitude *L. divaricata* seedlings were significantly smaller than low latitude *L. divaricata* seedlings before freezing, they had higher performance and survival following freezing. In the high latitude *L. tridentata* and low latitude *L. divaricata* populations mean seedling size pre-freeze was larger, but there was more variability in size in these populations, and larger seedlings lost more leaf area following freezing. This suggests that stabilizing selection may be operating in this population, since selection for increased seedling growth rate is generally considered to carry a fitness advantage (Dudley 1996).

Divergent selection for plant and/or leaf size in more benign vs. stressful environments has been observed in *Impatiens palida* (Bennington & McGraw 1995) and *Cakile edentula* var. *lacustris* (Dudley 1996), and in the woody species *Alnus sinuate* and *Betula papyrifera* where biomass accumulation decreased with increasing cold hardiness among plants originating from different latitudes (Benowicz *et al.* 2000). In a comparison of leaf size across high and low latitude one-year-old *L. tridentata* plants grown in the field and in a common garden, low latitude plants had significantly larger leaves than high latitude plants in the field ($P < 0.0001$) and in the greenhouse ($P < 0.0001$; J. Medeiros, *unpublished data*), a further indication that size may play a role in freezing tolerance. Leaf size also decreased with increasing elevation in both the field and greenhouse common garden populations of the tropical tree *Metrosideros polymorpha*, suggesting a strong genetic component (Cordell *et al.* 1998). Alternatively, seedling size may not be directly related to freezing tolerance, rather size may be correlated with other traits related to freezing tolerance, and there is strong evidence that correlated traits play a major role in the evolution of plant phenotypes (e.g., Conner & Via 1993; *reviewed in* Chapin III *et al.* 1993). Smaller seedlings may also have reduced growth due to increased investment in stress tolerance over investment in leaf area.

If fluctuating selection is responsible for the observed differences in freezing tolerance across seedlings of different sizes, we may expect to see that larger seedlings have higher survival following particularly warm winters, while smaller seedlings survive colder winters. Alternatively, there may be differences in the timing of seed production across maternal plants, with some maternal plants specializing in spring vs. fall seed production. If maternal families which produce seedlings with faster growth rates flower in the fall some years, spring seedlings could attain a greater size before winter with sufficient spring and summer precipitation. Once established, these larger seedlings may be able to withstand lower temperatures the following winter. In fact, freezing tolerance was higher among one-year old juvenile *L. tridentata* plants originating from the same high latitude site as the 3-month old seedlings included in the current study, and mortality is not observed in woody juveniles until minimum temperatures of -16^{o}C or below (*Chapter 2*).

Similarly, across 4 species of woody shrubs from the California chaparral seedlings were between 1-3^{o}C less freezing tolerant than woody adults (Boorse *et al.* 1998b). An eight year phenology study at the Sevilleta (our high latitude *L. tridentata* site) indicates that, although upwards of 80% of *L. tridentata* plants flowered in the spring and produced seeds in the fall, fall flowering is not uncommon, with 50% of plants flowering in the months of August and September in two out of eight years (Weatherill 2000-2008). In 2000, fall flowering was followed by a warm wet winter, resulting in a spring crop of seeds, with 20% of plants displaying new fruits in February and March of 2001. Fall flowering plants may effectively escape strong selection if they grow to sufficient size in their first year, making long-term minimum temperature more important for survival than average winter temperatures.

Our data provide evidence that freezing tolerance in seedlings is well coordinated with long-term minimum temperature, and we observed drastically reduced survival and post-freeze performance in low latitude *L. divaricata* seedlings just 2^{o}C lower than the long term minimum (-10^{o}C)**.** High latitude *L. tridentata* seedlings experience temperature of -10^{o}C several times per month during a typical winter and exhibited very little mortality at -10^{o}C and -12^{o}C**.** High latitude *L. divaricata* seedlings were intermediate in freezing tolerance. Though mean annual temperatures are similar for the high latitude *L. tridentata* site, there are no long term minimum temperature data for the high latitude *L. divaricata* site, so the relationship between freezing survival and minimum temperature is unknown. The relatively lower freezing tolerance of high latitude *L. divaricata* seedlings may be explained by the proximity of this population to the Atlantic coast which should dampen extremes, resulting in a higher long-term minimum temperature.

The observation that seedling mortality of *L. tridentata* occurs well below the long-term minimum temperatures at the site of origin, raises the question of when seedling establishment can occur in the field? In the Mojave a 13 year record of seedling establishment indicated that *L. tridentata* germinates after heavy late summer-early fall precipitation, but recruitment may also occur in the spring following rare but intense spring storms that occur perhaps only once per century (Beatley 1974). At the Sevilleta LTER, our high latitude *L. tridentata* site, establishment was monitored from 1988-2008 and during this time seedlings were observed only during 1999 (Moore 1989-2009). Weather data collected during the period of 1999-2000 indicate that the 1999 episode of seedling recruitment occurred following a summer with 1.5 times the long-term average precipitation. This was followed by a warmer than average winter (December 1999-February 2000 minimum temperature – 9.5^{o}C, average minimum winter temperature -11.8^{o}C; Moore 1989-2009) during which precipitation was low, only 34% of average for the months of October to February. In the subsequent year 2000, precipitation and temperature were average (276 mm precipitation January to December 2000 and a maximum summer of temperature 39^{o}C), and a survey of survival in late 2000 indicated that 92% of seedlings established in 1999 survived. These data confirm the assertion that establishment occurs during particularly wet periods, as well as provide some evidence that a warmer, drier winter is also crucial for survival.

A limitation of our experiment is that we are unable to distinguish the impact of maternal environmental effects on our seedlings, which are responsible if seed quality was affected or if seedling growth rates were reduced by the cost of expression of stress tolerance genes induced by the maternal growth environment. Three possible ways in which maternal environment could affect offspring performance and survival include: 1) differences in resource availability at the time of seed development, 2) differential maternal gene expression and 3) environmental effects on developing ovules, embryos or seeds (Mazer & Gorchov 1996). Effects of temperature in the maternal environment on seedling germination and growth have been detected within populations (Lacey 1996; Lacey & Herr 2000) and differences in seedling characters can be observed across maternal families (Alexander & Wulff 1985), across genetically identical lines (Wittle *et* *al.* 2009) and even across flowers on the same branch (Gutterman 1980). A temperature difference of 12^{o}C (10^{o}C vs. 22^{o}C) was sufficient to cause differences in seed mass and other seedling characteristics of *Arabidopsis thaliana* (Andalo et al. 1999). In such cases where parental plants are genetically similar, differential gene expression or selection on developing gametophytes could come into play (Case *et al.* 1996; Andalo *et al.* 1999). However, plastic responses of maternal plants to environmental cues can be genetically determined (Winn & Evans 1991), and the presence of maternal environmental effects does not eliminate the possibility that genetic differentiation has occurred.

The limited numbers of studies of maternal temperature effects have so far focused on differences in plant size, phenology and growth rate characteristics, and few have investigated effects of maternal environment on freezing tolerance of seedlings. A notable exception is Johnsen *et al.* (2005) in which changes in the timing of cold acclimation, bud set, de-hardening and bud set in the spring were associated with changes in gene transcription in seedlings of *Picea abies* experiencing cold vs. warm temperatures during embryogenesis. In our case, seeds and seedlings collected from the high latitude *L. divaricata* population were smaller and had been collected during a particularly cold spell. Given the complex nature of the relationship between plant size and freezing tolerance we observed, it is possible that not only differences in seed provisioning but differential gene expression across seedling populations was initiated by maternal environmental effects. Complex interactions between parental environment and seedling traits have been reported in studies of *Plantago lanceolata* (Lacey 1996; Wulff & Alexander 1985) and *Plantago asiatica* (Sawada *et al.* 1994).

The presence of maternal environmental effects may provide a fitness advantage if the timing and/or magnitude of stress is predictable, as was observed in the understory herb *Campanula americana* in which maternal light environment was a good predictor of that experienced by offspring (Galloway 2005). However, under field conditions, the fitness benefits of maternal environmental effects may be reduced by almost 40% when environmental heterogeneity is high (Lacey & Herr 2000). In the future, reciprocal transplant experiments of maternal plants followed by common garden studies of offspring should be conducted in order to resolve the extent to which maternal environment determines freezing tolerance under field conditions. Though our current study cannot distinguish maternal environmental effects, our data still provide pertinent information regarding the conditions conducive to establishment and survival of *Larrea* sp. at the three field sites.

CHAPTER FIVE

CONCLUSIONS

Climate is broadly considered to be one of the ecological variables with the greatest effect on patterns of establishment and survival and thereby, plant distributions (Comstock & Ehleringer 1992; Reyes-Diaz *et al.* 2005). The sporadic recruitment of annual and perennial arid land plants highlights the importance of precipitation and drought tolerance in controlling establishment (Barbour 1968). However, freezing tolerance had also been suggested as a factor limiting the high latitude establishment of the warm desert evergreen species *L. tridentata* (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002). Our goal was to elucidate factors that limit the distribution of *Larrea* species at high latitudes and better understand adaptations to freezing in this wide-spread evergreen genus. We determined the interactive effects of drought and freezing on plant performance and survival and the structure of variation within and among populations in physiological and morphological traits related to freezing.

WHAT ARE THE EFFECTS OF DROUGHT ON FREEZING TOLERANCE?

Both drought and freeze-thaw events can limit photosynthesis (Sakai & Larcher 1987), and reduce water transport capacity thereby causing early stomatal closure (Cochard & Tyree 1990; Sperry & Sullivan 1992; Tyree & Cochard 1996; Pockman & Sperry 1997; Langan *et al.* 1997). However, the freezing temperatures that damage leaves may not be the same as those that damage xylem (Feild & Brodribb 2001; Cavender-Bares *et al.* 2005). As plant water potential decreases, especially in drought-adapted species, solutes accumulate to maintain turgor. These same solutes are effective in reducing the freezing temperature of the cell sap and allow even greater leaf supercooling to at least -10^{o}C, while intercellular freezing within the plant apoplast and xylem occurs at temperatures closer to 0^{o}C (Sakai & Larcher 1987). If leaves are better able to withstand freezing during drought, and transpiration resumes following freezing at reduced water supply, hydration of the leaves may be impaired (Sperry *et al.* 2002).

However, if leaves are more vulnerable to lethal damage than xylem, as we observed in drought plants, this could effectively protect xylem from catastrophic embolism during subsequent drought by increasing the ratio of absorbing root area to transpiring leaf area. Frost-sensitive petioles in some species allow for leaf drop at temperatures too high to cause freeze-thaw xylem embolism (Sakai & Larcher 1987). Our data indicate that in the presence of drought, leaves are more vulnerable to freezing than drought, which effectively assures a higher ratio of transpiring leaf area to conducting xylem area.

In high latitude *L. tridentata*, drought and well-watered plants exhibit similar freezing responses following freeze-thaw events as cold as -8^{o}C. Some measure of increased freezing tolerance of living cells was observed at temperatures between -15^{o}C and -19^{o}C when drought was present. In addition, freeze-thaw embolism was maintained at rates which likely facilitated the greater retention of functional leaf area following freezing in drought compared to well-watered plants. We have also shown that *L. tridentata* is able to survive freezing temperatures near the long-term minimum temperature recorded at the Sevilleta LTER, but only in the presence of drought. Furthermore, climate change is predicted to shift water availability and temperature differentially, and wetter winters are likely to change the relative abundance of *Larrea* species across North and South America (Bachelet *et al.* 2001; Epstein *et al.* 2002; Peters 2002). Our data indicate that, in the absence of approximately 5°C increases in the long-term minimum temperature, increases in winter precipitation could reduce plant performance and survival at high latitude edges of *Larrea* distributions.

Our experiment has shed light on the whole plant effects of drought and freezing and the physiological factors involved in freezing tolerance in the genus *Larrea*. However, more work should be undertaken to determine the effects of more prolonged and/or extreme drought on freezing tolerance. Also, these results provide tantalizing evidence that freezing embolism may be prevented or repaired by living cells in *Larrea*, but we did not obtain any direct evidence of re-filling, leaving this as an important unanswered question. Finally, though it is clear that physiological changes during drought like solute accumulation increased freezing tolerance, we did not determine how gene expression was altered by drought and acclimation. Studies of gene expression could determine the mechanisms by which drought might increase freezing tolerance.

IS THERE EVIDENCE FOR LOCAL ADAPTATION TO FREEZING IN THE GENUS *LARREA*?

The performance and survival of individual plants under environmental stress is one factor that influences the structure of plant populations (Hutchings 1997), but the structure of genetic variation in physiological and morphological traits is necessary for local adaptation to take place. Differences in freezing resistance can be readily observed when comparing populations across the latitudinal range of a species (Sakai & Larcher 1987; Nardini *et al.* 2000; Cordero & Nilsen 2002), and there is evidence that populations growing at higher latitudes have higher acclimation potential than those at low latitudes (Gianoli *et al.* 2004). If carbon gain is limited by freezing in populations growing at the high latitude edges of warm deserts, then individuals with higher freezing tolerance, greater acclimation potential or even a more winter deciduous habit could have better fitness at ecotones. Selection acts on variation to produce phenotypes which can optimize carbon gain, thus plants should display adaptations to their local environment which allow them to maximize carbon gain, even in the face of interacting stresses like freezing and water limitation.

In order to better understand the role local adaptation might play in releasing plants from freezing limitation at the high latitudes, we tested the claim that genetic differentiation in freezing tolerance exists between low and high latitude populations of *Larrea* species. Both greenhouse common garden experiments indicate a genetic component to differences in freezing tolerance between populations of *L. divaricata* and *L. tridentata* sampled across latitude, and that divergence in this trait has occurred since the isolation of *L. tridentata* from *L. divaricata*. We detected significantly higher freezing tolerance in high vs. low latitude seedlings and the potential for the further evolution of this character. Wood characters indicated two strategies, both embolism avoidance and embolism tolerance, may increase overall plant performance and survival in high latitude populations. Particularly in *L. nitida*, embolism tolerance may allow plants to adjust dynamically to dramatic increases in water availability and demand that can occur in desert environments. Several interesting questions remain: first, does the timing of wood production and leaf drop differ among high and low latitude populations, and second, how plastic are wood characters in this genus?

The differences we observed across latitude for both *L. tridentata* and *L. divaricata*, in the absence of differences in ploidy, indicate that local adaptation to freezing has occurred in the warm desert shrub genus *Larrea*. In addition, the observation of two divergent approaches to wood structure in high latitude populations provides strong evidence that genetic differentiation is at least in part responsible for the differences we observed across latitude. We observed correlations between freezing tolerance and minimum temperatures accompanied by higher freezing tolerance in high latitude populations, suggesting trade-offs between increasing freezing tolerance and maximizing fitness in the absence of freezing. The evolution of increased freezing tolerance could have facilitated recent high latitude expansions of *Larrea* populations. Furthermore, the partitioning of variation in freezing tolerance along maternal family lines indicates that both high latitude *L. tridentata* and low latitude *L. divaricata* populations maintain the potential to respond to changes in temperature. The lack of family effects in the high latitude *L. divaricata* population may have resulted from prior selection for increased freezing tolerance and reduces the rate at which increased freezing tolerance would be expected to develop. Finally, fluctuating selection may maintain variability in seedling traits in the high latitude *L. tridentata* population, and more work needs to be done specifically to understand the various conditions under which seedling establishment occurs and how the maintenance of increased freezing tolerance could impact fitness when freezing stress is removed.

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